REGULATION OF LIPID ACCUMULATION IN GREEN ALGAE

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Contents

Abbreviations

APM	amiprophos-methyl
ARS	arylsulfurtase
CAP	chloramphenicol
CHI	cycloheximide
Chl	chlorophyll
C. kessleri	*Chlorella kessleri*
C. reinhardtii	*Chlamydomonas reinhardtii*
CT	control
DAG	diacylglycerol
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCW	dry cell weight
DGAT	diacylglycerol acyltransferase
DGDG	digalactosyl diacylglycerol
DGTS	diacylglyceryl-N,N,N-trimethylhomoserine
DGTT	diacylglycerol acyltransferase type two
DHAP	dihydroxyacetone phosphate
FAME	fatty acid methyl ester
FFA	free fatty acid
GPAT	glycerol-3-phosphate acyltransferase
GPDH	glycerol phosphate dehydrogenase
LPA	lyso-phosphatidic acid
LPAAT	lyso-phosphatidic acid acyltransferase
MAG	monoacylglycerol
MGDG	monogalactosyl diacylglycerol
LDs	lipid droplet
NLs	neutral lipids
PA	phosphatidic acid
PG	phosphatidylglycerol
PLs	polar lipids
RAD	regular air drying
SAC	sulfur acclimation
SE	standard error
SNRK	SNF1-related protein kinase
TLs	total lipids
TAG	triacylglycerol

General introduction

Lipids, which are hydrophobic components in organisms, are divided into polar lipids (PLs) and neutral lipids (NLs) by the chemical characteristics. PLs mostly form lipid bilayer to construct biological membranes with membrane proteins. Biological membranes not only prepare conditions for metabolism by separating internal environment from external one to limit traffic of substances, but also perform transportation of materials or provide the place in which membrane proteins function.

Membrane lipids, which comprise biological membrans, do not have any activities like those of proteins. Respective biological membranes show characteristic lipid compositions. This characteristic suggests that lipids of biological membranes contribute to the exertion of membrane functions. For example, thylakoid membranes, which perform photosynthetic reaction, include four classes of lipids : monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl giacylglycerol (SQDG), phosphatidyl glycerol (PG) (Fig. a). These lipids are commonly found in O_2 evolving photosynthetic organisms (Gounaris et al., 1986). Photosystem II (PSII) protein complexes involve these lipids (Lu et al., 2007). Our laboratory reported that SQDG in thylakoid membranes contributes to the maintenance PSII structure and activity (Sato et al., 1995a; Aoki et al., 2004). Moreover, SQDG functions as sulfur pool for protein synthesis in *C. reinhardtii* under sulfur starved conditions (Sugimoto et al., 2007). Thus, SQDG plays a role in supporting PSII activity and providing sulfur as intracellular-sulfur reservoir.

Melting points of fatty acids included in membrane lipids are changed by the length of carbon chains, number of double bonds (degrees of unsaturation), and the cis/trans type of the double bonds. Shorter carbon chains or, in particular, higher degrees of unsaturation generally decrease the melting point. For example, when palmitic acid (16:0) turns into palmitoleic acid (16:1), the melting point declines from 63.1°C to -0.5°C (Table a). This change is caused by molecular density of fatty acids, and vacant space increases fluidity among lipids. It was reported that, in cyanobacteium *Synechocystis* sp. PCC 6803, fatty acids were desaturated in membrane lipids without de novo fatty acid synthesis when growth temperature was shifted from 38°C to 22°C (Wada et al., 1990). It is considered that the regulation of membrane fluidity is necessary for the proper functioning of biological membranes (Cossins et al., 1989)

NLs include triacylglycerol (TAG, Fig. b), diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acids (FFA), sterols, and carotenoids etc. NLs do not construct lipid bilayer membranes, but their functions are necessary for cell metabolisms. For example, DAG, which is an intermediary metabolite in synthesis of membrane lipid and TAG, is known to work as one of the second messengers. DAG is produced from phospholipids such as phosphatidylinositol by phospholipase C, and then activates the protein kinase C. As to other

NLs, carotenoids are pigments including β-carotene, lutein, and astaxanthin, which respectively have a potent anti-oxidative activity. β-carotene is also known as a precursor of vitamin A. However, the amount of NLs mainly depends on TAG content in cells. TAG is synthesized mainly on the Kennedy pathway, through transfer of three fatty acids to 3-phosphate (G3P) (Fig. c). The fatty acids released from TAG after lipase digestion are used for β oxidation to yield acetyl-CoA. Acetyl-CoA is used for production of chemical energy sources such as ATP in TCA cycle, or it is changed to carbohydrates by glyoxylate cycle. The fatty acids also take part in PLs synthesis. Thus, TAG can be regarded as storage lipid of energy and carbon.

TAG is included widely in animals and plants. Animals such as human and mouse hold adipocytes which synthesize and reserve TAG, while plant accumulates TAG in specific organs such as seeds and fruits. TAG form lipid droplet (LDs) which is surrounded by monolayer of phospholipids (Tzen et al., 1992).

Algae are eukaryotic photosynthetic organisms, which are classified into various groups such as *Chlorophyta* (green algae), *Bacillariophyta* (diatomea), *Phaeophyta* (blown algae), *Rhodophyta* (red algae), etc. Algae also have TAG as lipid droplets (LDs). Interestingly, algae accumulate TAG under some stress conditions. In green algae, it is well known that cells increase starch and lipids (especially TAG) content in nitrogen (N)-deplete cultures. In diatomea which have outer shells made of silica (Si), TAG accumulation is observed under Si-deprived condition (Chen et al., 2008, Eizadora et al., 2009). However the reason of algal TAG accumulation under stress conditions is still unclear.

TAG from plants is utilized for production of food oils and processed goods. Moreover, attention is recently paid to production of biodiesel fuel (BDF) from plant oils from the aspect of carbon neutral conception or green sustainable chemistry. Production of biomaterial from algae have advantages such as effective use of land for culturing, and high yields in short term, for instance, so that the applied algal technology for production of biomaterials from algae have been attempted actively in the world. However, there still remain many technological problems. The author has reported about membrane lipid changes as a response to sulfur deficient stress in microalgae. In the process of lipid analysis, it was found that green alga *Clamydomonas reinhardtii* (*C. reinhardtii*) accumulates TAG under sulfur deprived condition. The information on the mechanism for algal lipid accumulation is limited. Therefore, lipid changes and gene analyses were performed to investigate TAG accumulating mechanisms in algae.

Green alga, *C. reinhardtii*, which has a bowl shaped chloroplast, is a unicellular photosynthetic organism (Fig. d). Growth by asexual reproduction is possible, and many molecular biological methods have been established as one of model organisms. The nucleotide sequence of nuclear genome was determined in 2007 (Merchant et al., 2007).

Various mutants have been isolated and analyzed so that there are abundant genetic findings about various physiological functions.

Table a. Major fatty acids and each melting point

Fatty acid	Common name	Melting point (℃)
12 : 0	Lauric acid	44.2
14 : 0	Myristic acid	52
16 : 0	Palmitic acid	63.1
18 : 0	Stearic acid	69.6
20 : 0	Arachidic acid	75.4
22 : 0	Behenic acid	81
24 : 0	Lignoceric acid	84.2
16 : 1	Palmitoleic acid	-0.5
18 : 1	Oleic acid (*cis*-Δ^9)	13.4
18 : 2	Linoleic acid (*cis*,*cis*-Δ^9,Δ^{12})	-9
18 : 3	α-Linolenic acid	-17
18 : 3	γ-Linolenic acid	
20 : 4	Arachidonic acid	-49.5
20 : 5	Eicosapentaenoic acid (EPA)	-54
22 : 6	Docosahexaenoic acid (DHA)	-44.

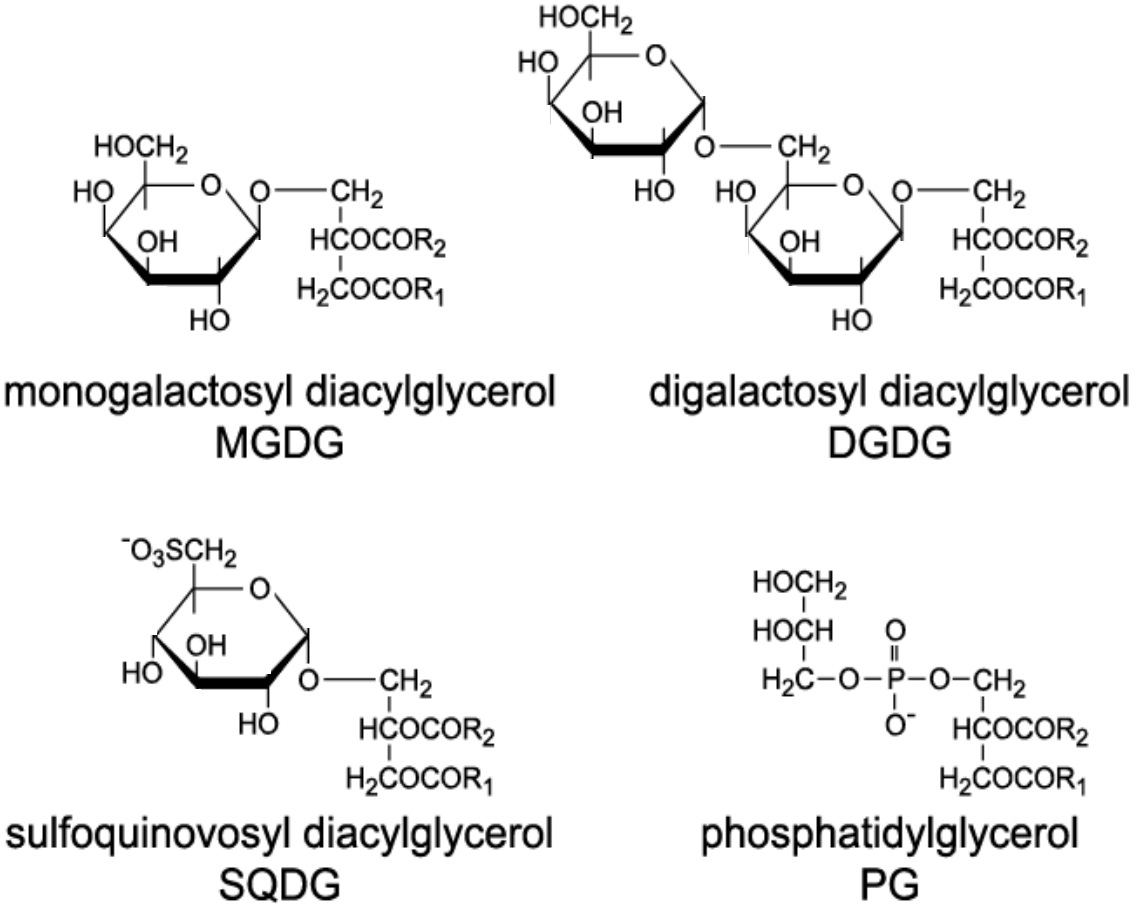
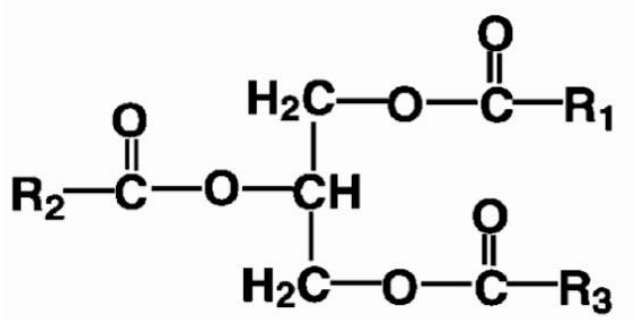
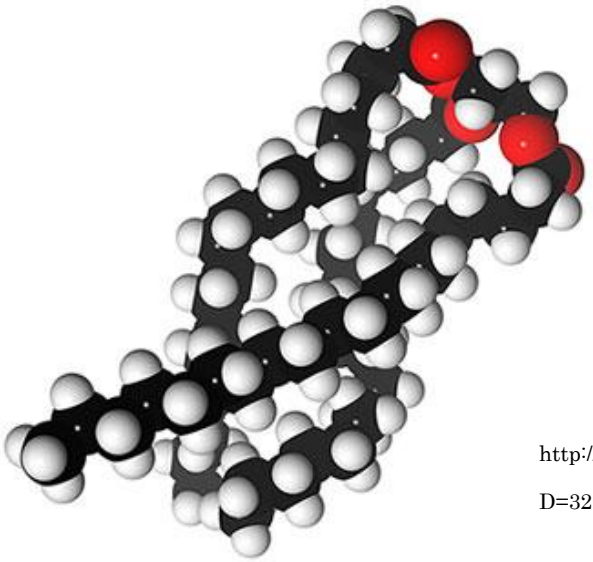


Fig. a. Thylakoid lipids. Monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulfoquinovosyl diacylglycerol (SQDG), are galctolipids. SQDG and phosphatidylglycerol (PG) contain sulfur and phosphorus in their structure, respectively and have minus charge.





http://www.3dchem.com/moremolecules.asp?ID=320&othername=triacylglycerol

Fig. b. Structures of triacylglycerol (TAG). Fatty acids combine at R position by ester bond.

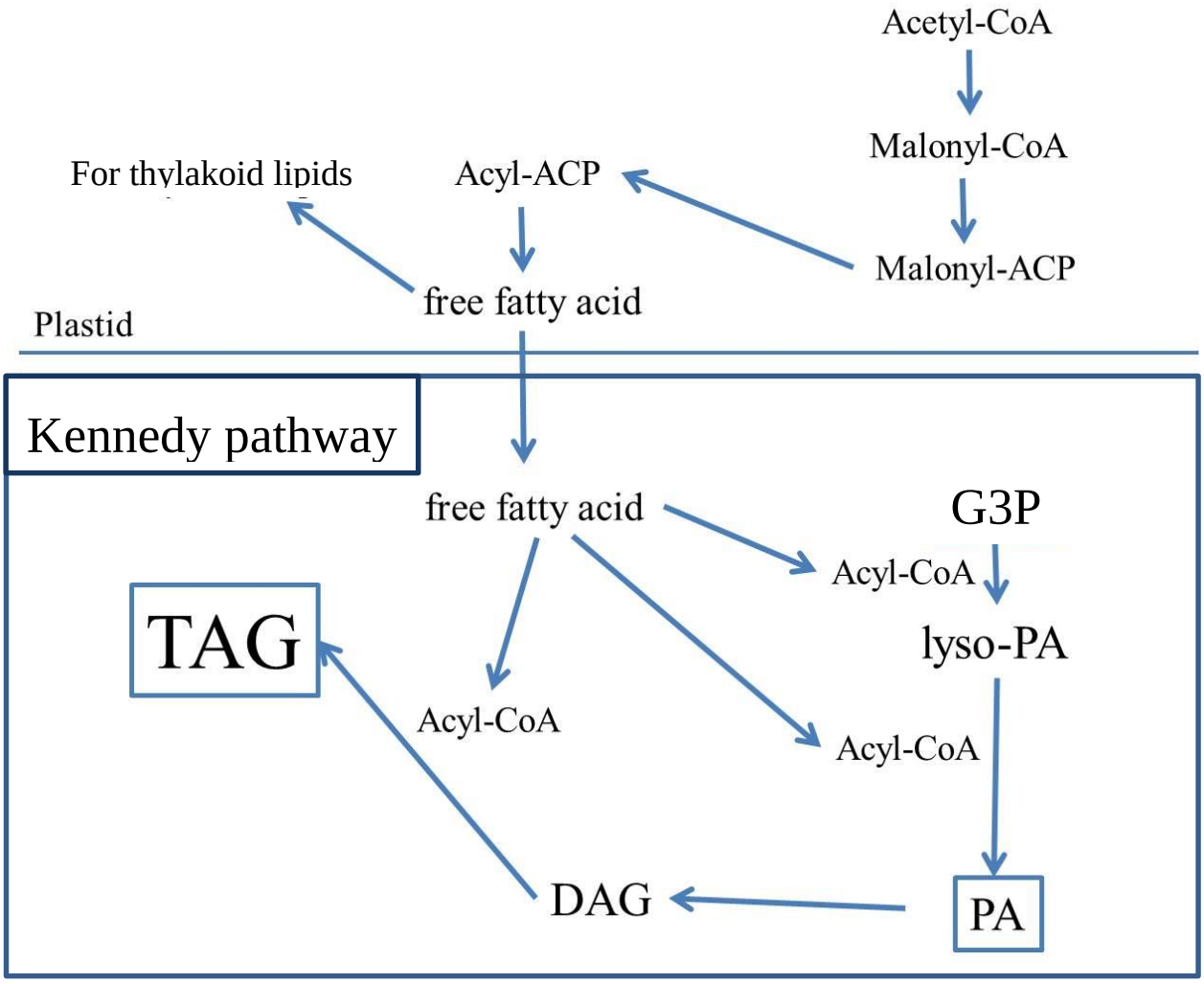


Fig. c. TAG synthesis is carried on Kennedy pathway from glycerol-3-phosphate (G3P) to TAG via lyso-phosphatidic acid (PA), PA and DAG.

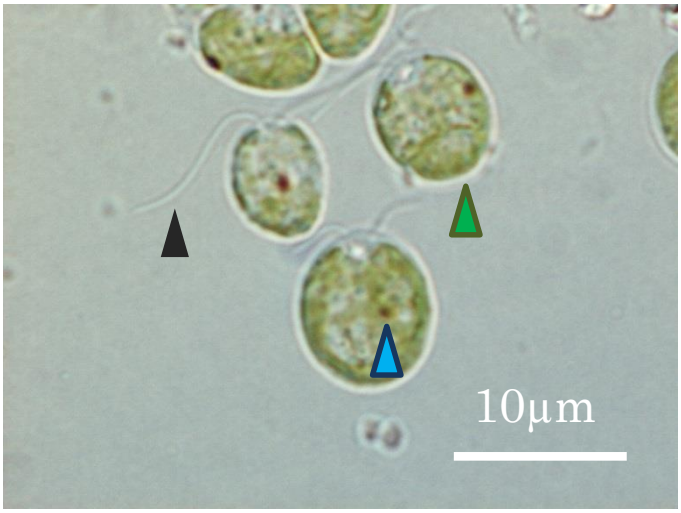


Fig. d. *Chlamydomonas reinhardtii* CC125 mt+
C. reinhardtii can swim using flagella (black triangle) forward to light. Eye spot (blue triangle) works as sensor of light. Contains a bowl shaped chloroplast (green triangle) and cell wall (no visual).

Chapter 1

TAG accumulation in green algae

1-1 Introduction

Triacylglycerol (TAG), which is one of the neutral lipids, is ubiquitous in eukaryotes and also present in a limited group of prokaryotes (Alvarez et al., 2002). Distinct from PLs that are predominantly membrane components, TAG has formed as lipid droplets (LDs). TAG has been considered as a storage compound: *e.g.* in oil seed plants, FAs esterified to TAG in seeds utilized by β-oxidation for energy production and gluconeogenesis, which supports the seedling growth after germination (Stone et al., 1997, Quettier & Eastmond, 2009). Recent studies indicated that TAG also participates in the synthesis of membrane lipids as an intermediate metabolite by supplying FAs in actively growing cells of yeast, *Saccharomyces cerevisiae*, and thus is critical for maintenance of lipid homeostasis (Rajakumari et al., 2010; Kohlwein & Henry, 2011). On the other hand, the view of industry, TAG is important as food oil and has been recently expected as a source of biodiesel fuel (BDF), which is produced through its chemical conversion into methyl or ethyl esters of FAs. Extensive attention has been paid to BDF production with photosynthetic organisms in particular, in terms of the carbon neutrality concept (Hu et al., 2008).

Many algal species have several advantages over terrestrial plants as to the production of biomaterials, including high annual biomass productivity on an area basis that is ensured by their high growth rates (Hu et al., 2008). Eukaryotic algae generally contain TAG at a low level during optimal growth conditions, but the content remarkably increases along with aging of the culture or under unfavorable stress conditions such as nutritional limitation (Hu et al., 2008), high salinity (Siaut et al., 2011), or high light (Picaud et al., 1991). As to nutrients, nitrogen deficiency has been shown to be the most effective factor for induction of accumulation of TAG in numerous algal species including green and red algae, diatoms, golden algae, haptophytes, eustigmatophytes, dinophytes, and yellow-green algae (Hu et al., 2008). Study of the mechanism by which the accumulation of TAG is stimulated, e.g. identification of key genes as to TAG synthesis and proteomic analysis of LDs, is indispensable for enhancement of the productivity of TAG, but this has only just begun for algal species (Nguyen et al., 2011; Boyle et al., 2012; Deng et al., 2012; Msanne et al., 2012).

The author had studied about thylakoid membrane lipid changes under nutrient such as sulfur and phosphorus, deficient conditions using green alga *C. reinhardtii*. On that process of lipid analysis, it was found that *C. reinhardtii* accumulates NLs in sulfur-starved condition. NLs were nearly occupied by TAG in general. Therefore, firstly, the author demonstrated TAG accumulation under sulfur deficient condition, and performed quantitative analysis to compare with TAG increasing in known by nitrogen deficiency in this chapter.

In addition to nutrient-deficiency derived TAG accumulation, this study describes an interesting cultivating method in algae. Our laboratory wrestles with algal cultivating systems using flat cloth. Swabbing cells to solid surface of cloth omits to remove the culture liquid to harvest cells, so that reduce the cost. To understand cell behavior on solid surface, growth and lipid changes were measured in *C. reinhardtii*.

1-2 Material and Methods

Strains and growth conditions

C. reinhardtii and *Chlorella kessleri* 11h were cultured with continuous illumination at 30°C in TAP medium (Harris, 1989) for mixotrophic growth of *C. reinhartdii* cells in a flask on a rotary shaker, or in 3/10 HSM (Gillham et al., 1970) for photoaoutotrophic growth of *C. reinhardtii* and *C. kessleri* cells in an oblong glass vessel with aeration. For transfer to nutritionally starved conditions, cells grown to the mid-logarithmic phase in TAP medium or ones grown to the mid-linear phase in 3/10 HSM were harvested by centrifugation, washed twice and then resuspended in the corresponding S-, N- or P-free medium (Sugimoto et al., 2010). Growth of the cells was monitored by determination of the optical density at 730 nm with a spectrophotometer DU800 (Beckman, USA). When needed, the flask, in which cells of *C. reinhardtii* were mixotrophically cultured, was completely covered with aluminum foil for subjection to the dark condition.

Extraction of lipids, and their quantitative analysis

Total lipids were extracted from cells of *C. reinhardtii* and *C. kessleri*, respectively, according to the method of Bligh and Dyer (1959), and thereafter separated into individual classes of neutral lipids by TLC on precoated silica gel plates [Merck 5721, (Sugimoto *et al.*, 2007)] with a solvent system of hexane/diethylether/acetate (70:30:1, v/v/v, Chen et al., 2008). The spots of lipids were visualized by illumination of UV light after spraying with primulin (0.01% in 80% acetone, w/v). Fatty acid methyl esters were prepared from the total lipids, TAG, and free FAs by heating at 95°C with 5% anhydrous methanolic HCl, and thereafter analyzed by capillary GLC, as described previously (Sato *et al.*, 1995). The FA content of each fraction was estimated with arachidonic acid as an internal standard.

Microscopic observation of lipid droplets

A Nile red solution (0.25 mg·ml^{-1} in acetone, Keith et al., 1987) was added to respective cell suspensions of *C. reinhardtii* and *C. kessleri* (1:50, v/v) and the stained cells were observed under a fluorescence microscope (BX-FLA, Olympus Optical Co., Tokyo, Japan) with the use of a 520-550 nm excitation filter.

Regular air drying condition and harvesting

To impose regular air-drying (RAD) condition on the cells, 20 mL of a pre-culture was vacuumed on a glass fiber filter (GF/C 47mm, Whatman GE healthcare UK) which were measured the weight in advance. Thereafter, the cells were incubated on the filter at 30°C for 96 h in a plastic box with illumination with fluorescent light (15 μmol photons/m^2 s). For slow drying of the filter, the air was circulated with an air pump with as high as 99% humidity, and at a rate that allowed the air in the chamber to be replaced with in one hour. On the other hand, for less severe air-drying, the glass filter on which the cells were layered was put on a metal mesh (mesh size, 1mm^2) over a filter paper soaked

in culture medium or H_2O. For measurement of dry cell weight (DCW), the cells on the glass filter were placed in an oven dryer (FS-420. Advantec, Tokyo) at 55°C for more than 5 h. Since there was no significant effect of this heat treatment on the weight of a glass filter itself (data not shown). Thereafter, subtracting premeasured filter weight from total weight.

1-3 Results

Enhanced accumulation of TAG under stress conditions in C. renhardtii

Growth rates were measured in *C. reinhardtii* under TAP, TAP-S (-S), TAP-N (-N), TAP-P (-P) (Fig. 1-1). Cells under TAP medium increased to 5.1-fold as a maximum level at 72h, and the value was kept until 120h. In contrast, under stress condition -S, -N, -P showed 2.1, 1.8, 2.9-fold respectively at 120h. Chlorophyll (Chl) content was also suppressed in each medium, and especially in TAP-S and TAP-N, Chl decreased to less than a half of initial level (Fig. 1-2).

LDs were increased under -S, and -N conditions remarkably, but LDs were observed generally in cells (Fig. 1-3). Stress exposed cells tended to become corpulence comparing with control, and popping cells were often observed (see in -N). Moreover it was investigated that iron (-Fe), and both nitrogen and phosphorus (-N, -P) deficient stress. As a result, only double -N, -P conditions led increase in LD, although cell bleaching seen in a few cells.

Lipids were extracted from cells under stress conditions, and were separated into neutral lipids class (FFA, TAG) by TLC, and exhibited TAG accumulation under -S and -N conditions. TAG was also included in -P cells (Fig. 1-4).

TAG content and fatty acid composition

FAs analysis about TAG, FFA, and total lipids (TLs) were performed by gas chromatography. TAG content relative TLs were 40.3% as the highest volume at 72h under -S condition. After that, the volume decreased gently, and showed 36.3% finally at 120h. In the case of -N condition, TAG content kept increasing until 120h, and reached 56.6%. While -P conditions, TAG content declined to 2.3% after 24h from 7.7% at the beginning time. After that, it recovered slowly to 5.6% at 120h (Fig. 1-5). TAG content relative to the liquid culture were shown by adjusting TLs content at 0h to 100μM (Fig. 1-6). TLs contents increased under -S, -N, -P conditions, and showed 330.4μM, 287.8μM, 220.1μM respectively. TAG content were 98.3μM、166.8μM、12.2μM. In these result, it was suggested that -S conditions causes TAG accumulation as well as in known N-limitation, but P-limitation have no relationship to TAG accumulation in *C. reinhardtii*.

FAs composition were common in each -S, -N, -P (table 1-1, 2, 3). TAG possessed palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3) mainly. By stress condition degrees of unsaturation was raised but no change in C16/C18 ratio (table 1-4).

Photo autotrophic TAG accumulation in algae

To understand TAG accumulation in photo autotrophic (PA) growth, *Chlorella kessleri* (*C. kessleri*) and *C. reinhardtii* were brought up with 3/10 HSM medium. Growth rate in *C. kessleri* was suppressed to 0.43 and 0.28-fold under PA -S, and -N respectively (Fig. 1-7). PA -S growth showed 3.5 -fold in *C. reinhardtii*, the value was as 1.6-fold high as that of mixo trophic -S (MX: means TAP-S growth) growth (see Fig. 1-1). Cells of *C. kessleri* contained large LDs, but not became corpulence like *C.*

reinhardtii (Fig. 1-8), and increased TAG content, that shown in TLC (Fig. 1-9).

TAG contents in the PA growth condition were shown in Fig. 1-10. Comparing MX and PA under -S condition in *C. reinhardtii*, PA condition raised TAG content relative to TLs at 56.9% which was nearly equal to MX-N condition. *C. kessleri* accumulated TAG at 43.5%, and 73.0% in -S, and -N respectively. There were no differences between *C. kessleri* and *C. hardtii* in TAG accumulation under -S condition. However, TAG production per liquid culture in *C. kessleri* was more twice higher than that in *C. reinhardtii*.

PA growth did not affect FA composition of TAG and TLs in *C. reinhardtii* (table 1-5) *C. kessleri* included palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid mainly. Especially, linoleic acid occupied about 30% to as the whole of TAG (Fig. 1-1)

Change of fatty acid composition by temperature in C. kessleri

C. kessleri grew at 35°C as well as 30°C, and increased 5.6-fold at 120h under -S condition. However, vaporization of culture medium is notably so that values in A_{730} were varied. The other one at 20°C showed 4.1-fold (Fig. 1-12).

Interestingly, TAG contents relative to TLs were 78.8% and 52.5% at 20°C and 35°C under -S condition (20°C-S, 35°C-S) respectively (Fig. 1-13a). These values are higher than the value of 30°C-S as a usual condition. Lipid contents relative to cell volume were shown in Fig. 1-13b. The values of TAG were 345μmol and 291μmol in 20°C-S and 35°C-S conditions. These values were 2.1 and 1.8-fold higher than that of in 30°C-S condition (Fig. 1-13b).

FA composition of TAG changed remarkably in 20°C -S condition. Stearic acid and linoleic acid decreased, but oleic acid and linolenic acid increased. Especially oleic acid was contained more than 40% in TAG (Fig. 1-14). Degree of unsaturation was increased to 1.41 from 1.23, and melting point was decreased to 11.9 degrees Celsius (table 1-6).

Comparative analyses of TAG accmulation in C. reinhardtii and C. kessleri

Each strain were suppressed growth rate under -S condition, but *C. kessleri* showed more than 5-fold increase which was higher than that of *C. reinhardtii* in TAP medium (Fig. 1-15a). TAG accumulations were observed from 24h and 48h in *C. reinhardtii* and *C. kessleri* separately. There were no significant differences in final TAG content relative to TLs and cell volume, interestingly (Fig. 1-15b,c). For example, TAG content relative to TLs were kept 40±3% in each strain. However, produced TAG volume in the culture was 371μM in *C. kessleri*. It is 4.8-fold higher than that of *C. reinhardtii* (Fig. 1-15d).

TLs content relative to dry cell weight (DCW) were shown in Fig. 1-16. *C. reinhardtii* contained lipids 8.5% in generally, and increased 14.9% and 17.5% under -S and -N conditions. On the other hand in *C. kessleri*, it was 18.4% and 34.0 % under photo-autotrophic -S and -N condition. It couldn't

compare with *C. reinhardtii* and *C. kessleri* simply because of difference of culture medium, but there was found that -N condition tended to cause high accumulation of TAG.

Air-drying cell growth and TAG accumulation in C. reinhardtii

Cells were grown on glass-fiber filter aerating 2% CO_2 in chamber, and named this condition regular air drying (RAD). RAD condition was able to grow the cells till 48h but decreased Chl content to a half of initial level at 120h (Fig. 1-17). LDs were contained in RAD cells but half of these cells were died (Fig. 1-18a). TAG volume in which was 37.9% at 72h as the highest level, kept until 120h with slow decreasing (Fig. 1-18b). FA composition of TAG was C16 saturated FA and C18 unsaturated FAs as well as that of other conditions (table 1-7).

To demonstrate what determined TAG content under RAD condition, culture medium (3/10 HSM) or pure-water (H_2O) were supplied from the bottom of filter (see supplement Fig. 1-a). Growth rate and TAG content were measured (Fig. 1-19). Supplying 3/10 HSM allowed higher growth and Chl content than supplying H_2O or RAD condition (Fig. 1-19a). About TAG contents, supplying H_2O showed 1.4-fold as high as RAD condition, in contrary, supplying 3/10HSM decreased to 0.8-fold. TAG contents relative to dry cell weight (DCW) were 4.9%, 3.2%, 5.9% in supplying 3/10HSM, supplying H_2O, and RAD respectively (Fig. 1-19b).

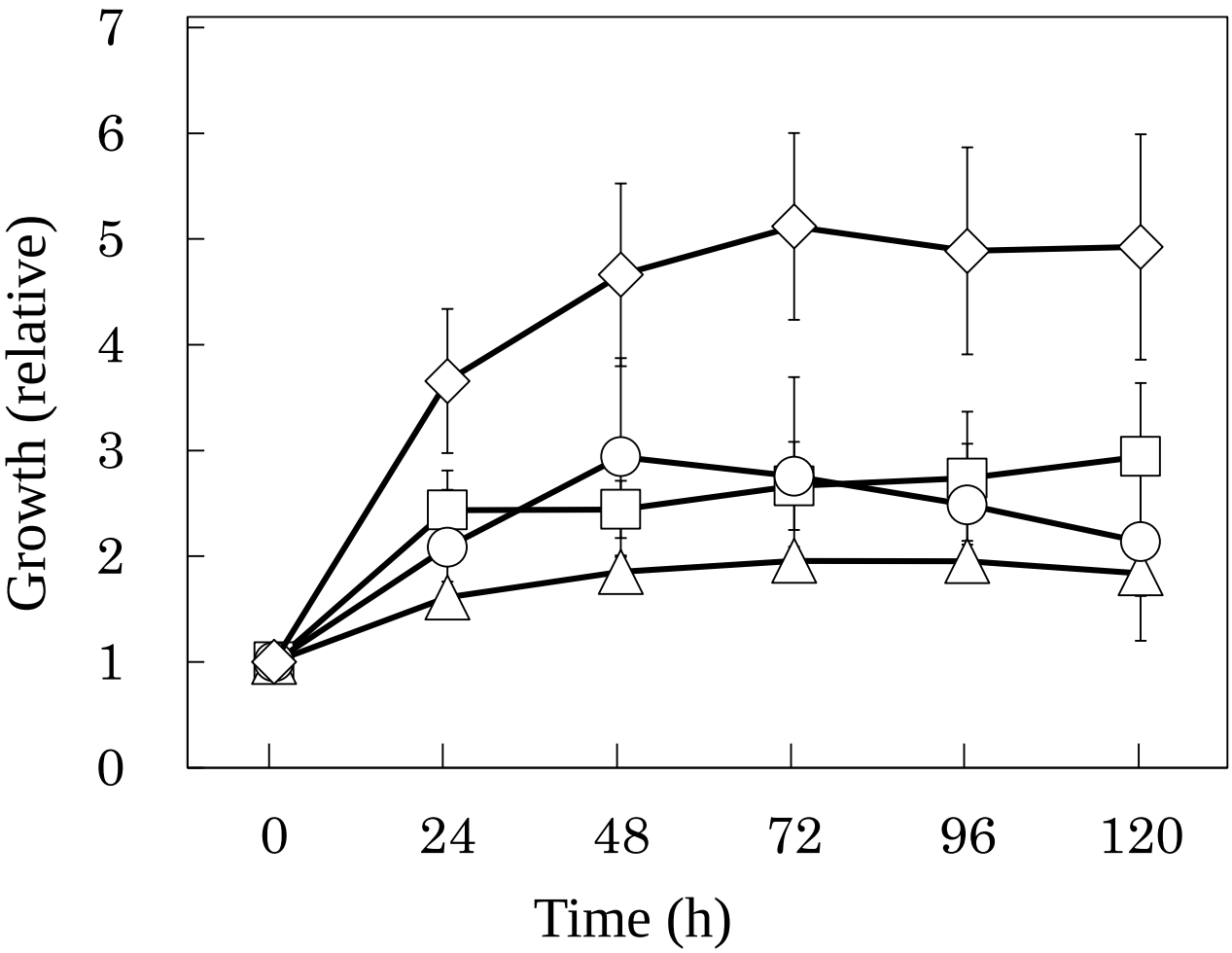


Fig. 1-1. Growth rate under TAP (open diamonds), TAP-S (open circles), TAP-N (open triangles), and TAP-P (open squares) in *C. reinhardtii*. OD_{730} values were measured and set value at 0h to 1.0 as initial level in each condition. Three experiments were performed and the values are the averages ± SE.

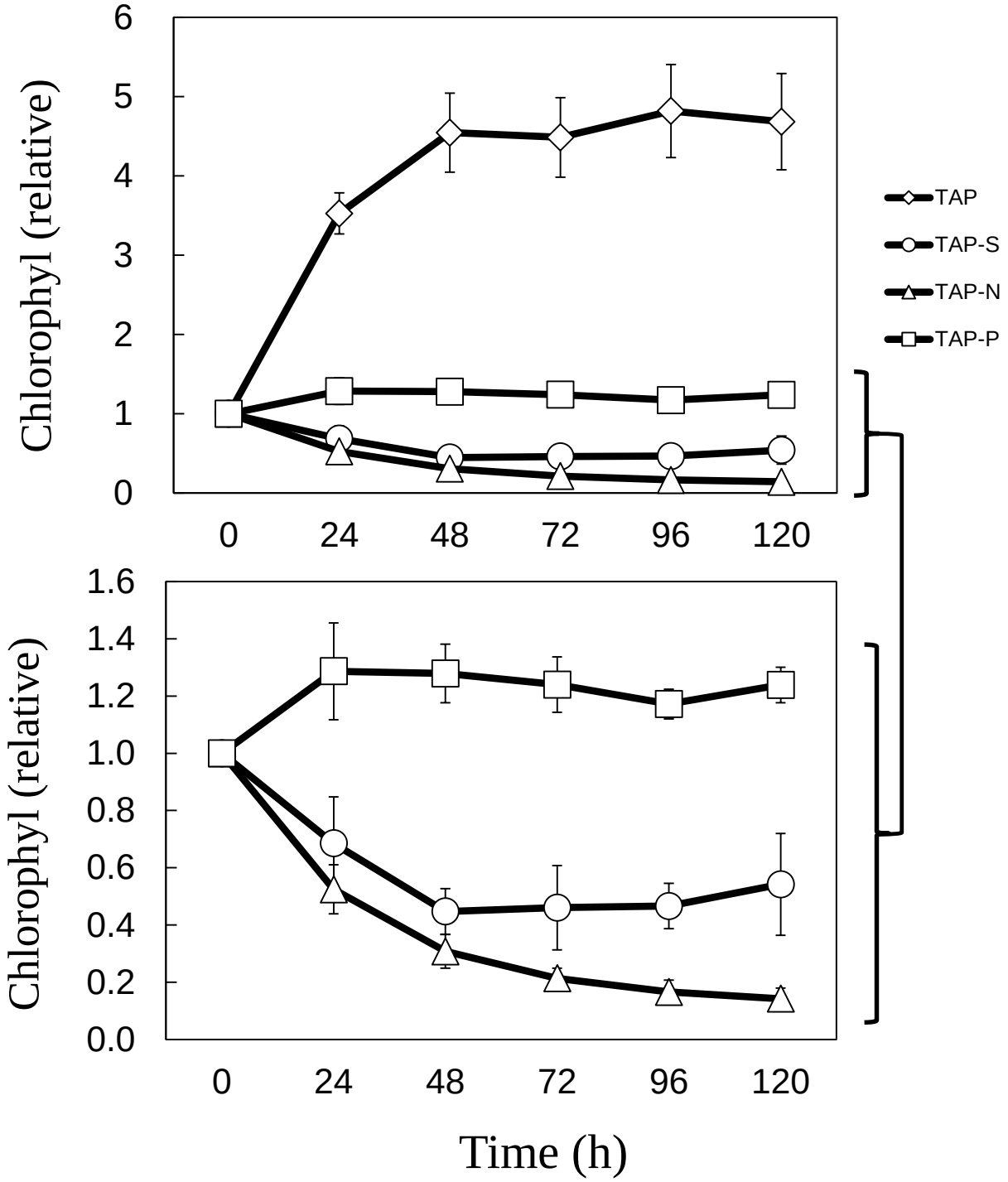


Fig. 1-2. Chlorophyll contents under TAP (open diamond), TAP-S (open circles), TAP-N (open triangles), and TAP-P (open squares). Values were shown relative to 0h and the averages ± SE determined by three independent experiments.

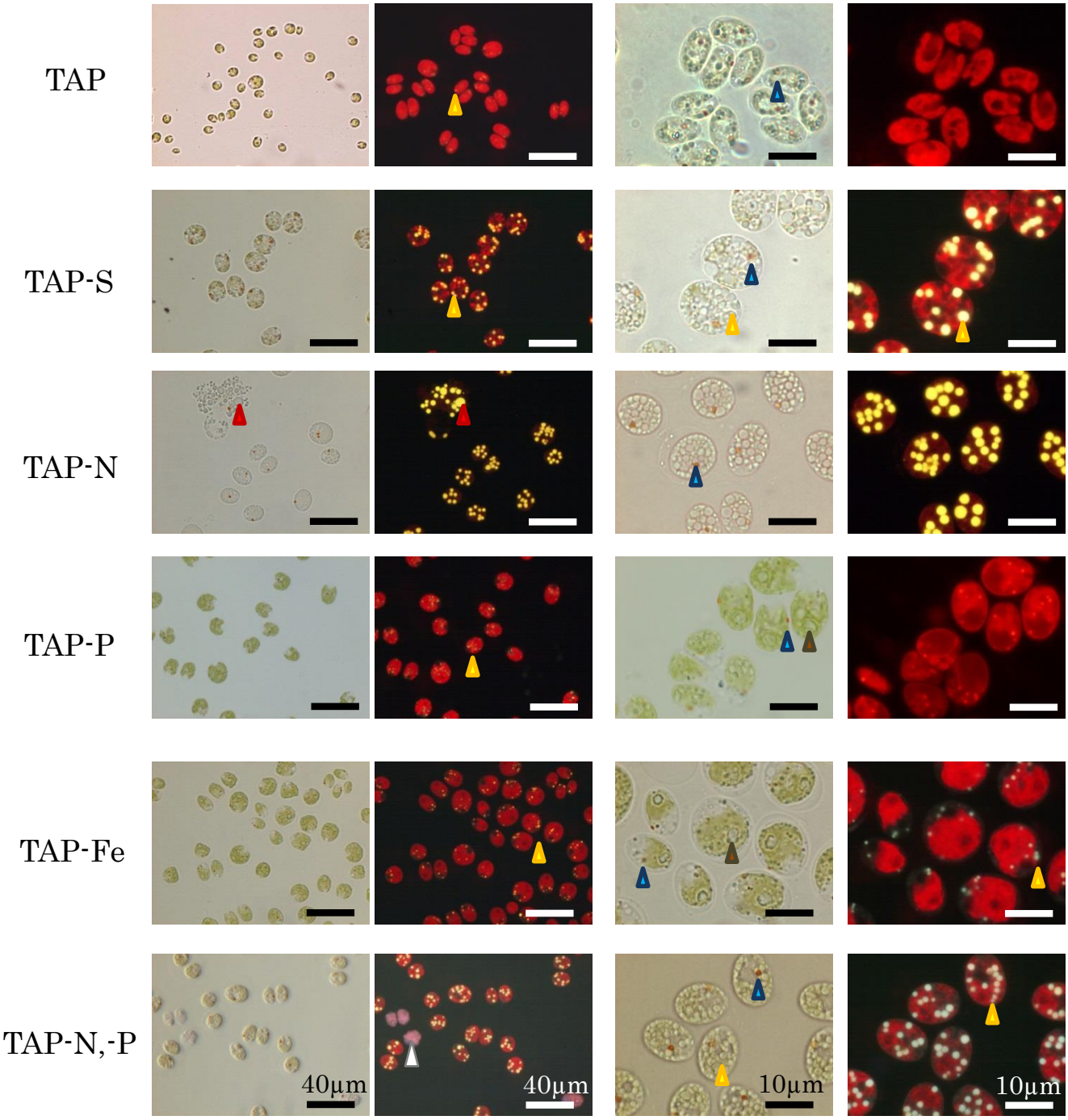


Fig. 1-3. Cells under various conditions were observed by microscope. Cells had eye spot (blue triangles), and lipid droplets (LDs, yellow triangles). Cells swelled, and popped out including LDs (red triangles). Cells formed pyrenoid (brown triangles). Some cells were bleached (white triangles) in both nitrogen and phosphorus deficient culture TAP-N,-P. LDs were stained by Nile Red and observed under excitation light (520-550nm)

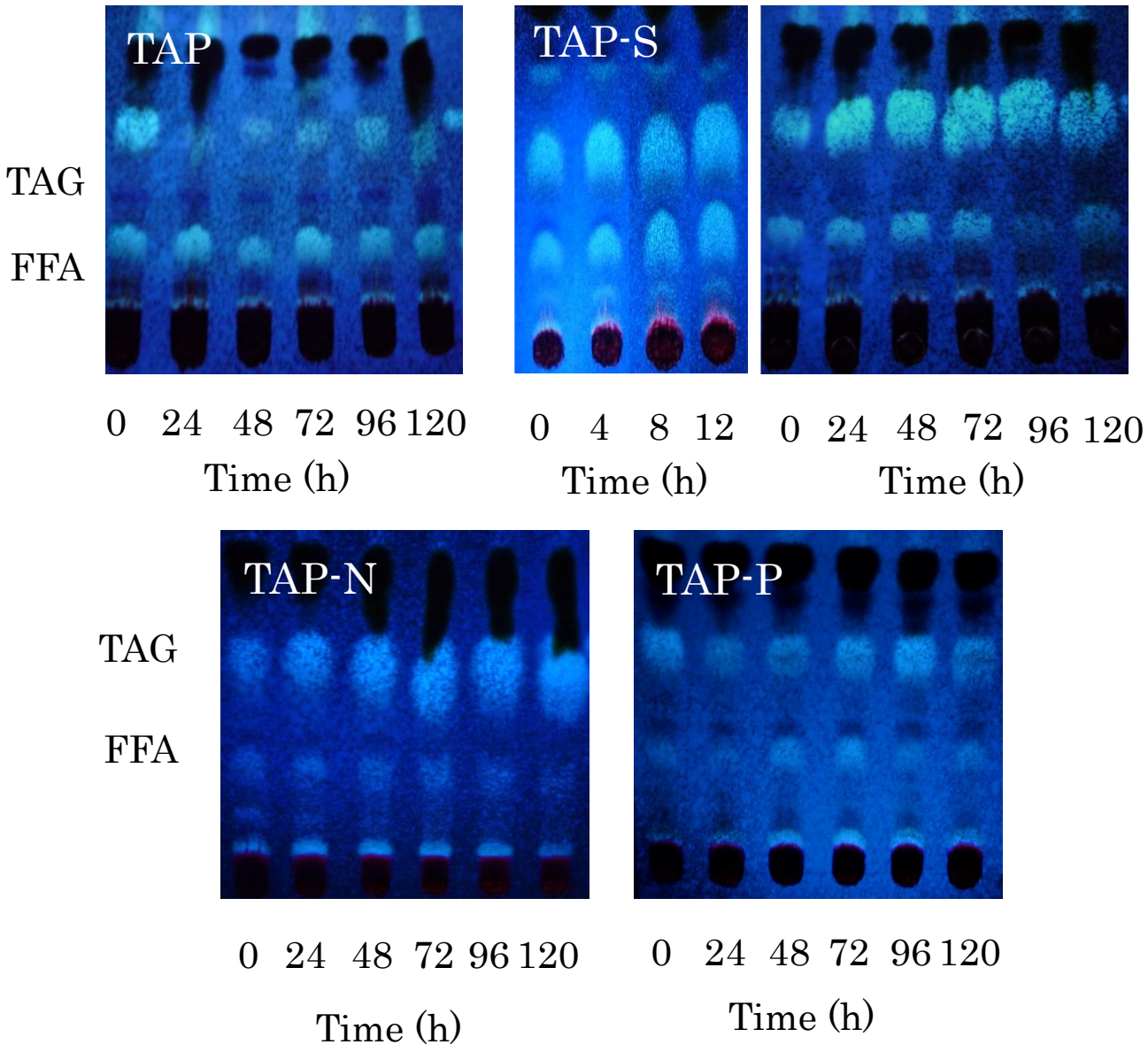


Fig. 1-4. Separation of neutral lipids by TLC with a solvent system of hexane/diethylether/acetate (70:30:1, v/v/v). Neutral lipids include TAG, DAG, MAG, FFA, ester, and carotenoids. Lipids in each lane were extracted from 30ml culture of cells, respectively.

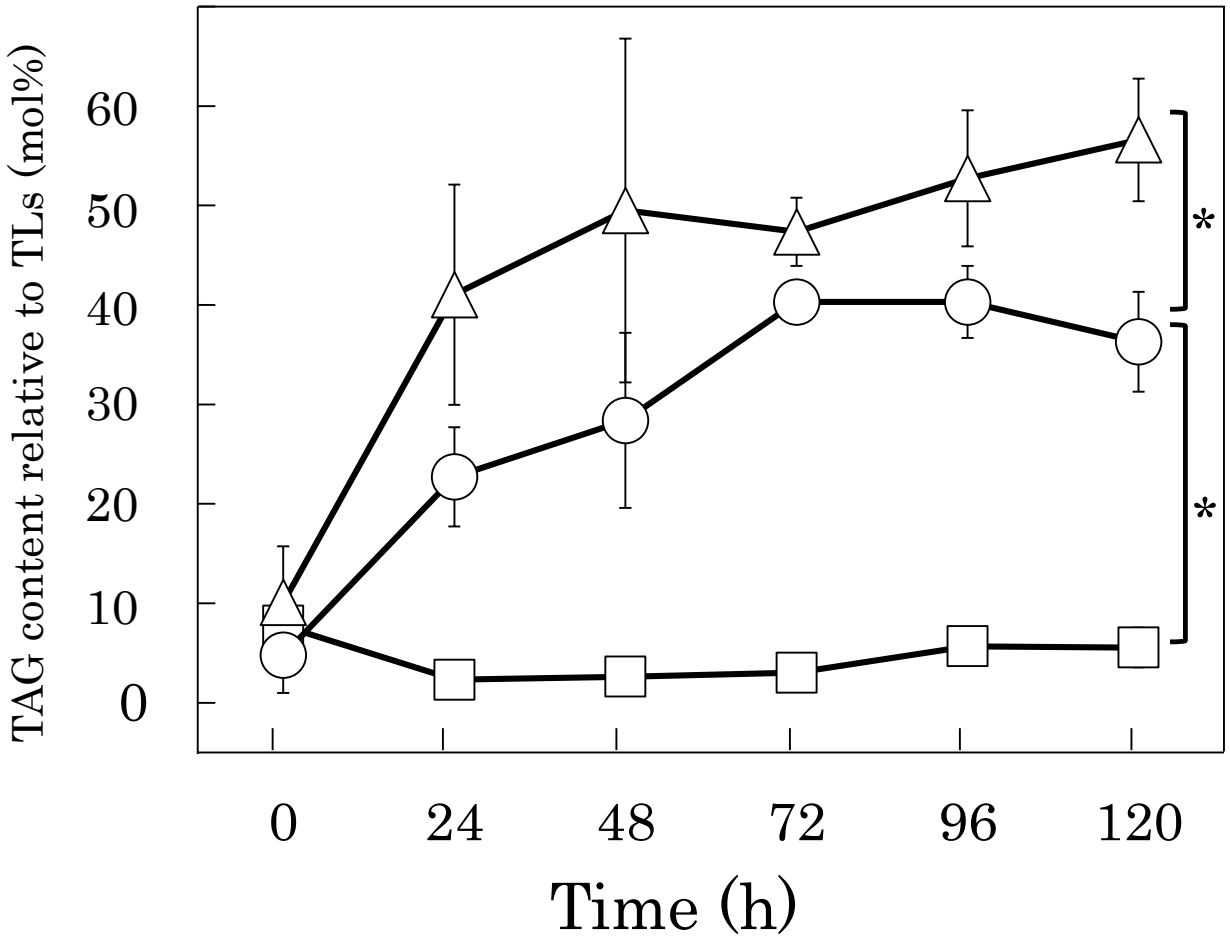


Fig. 1-5. TAG amount relative to TLs in TAP-S (open circles), TAP-N (open triangles), TAP-P (open squares). Fatty acids from TAG or TLs were formed to fatty acid methyl ester (FAME), and analyzed by GC with internal standard. All values mean average ± SE determined by three independent experiments. Asterisk means that there was meaningful difference between two results ($p < 0.05$).

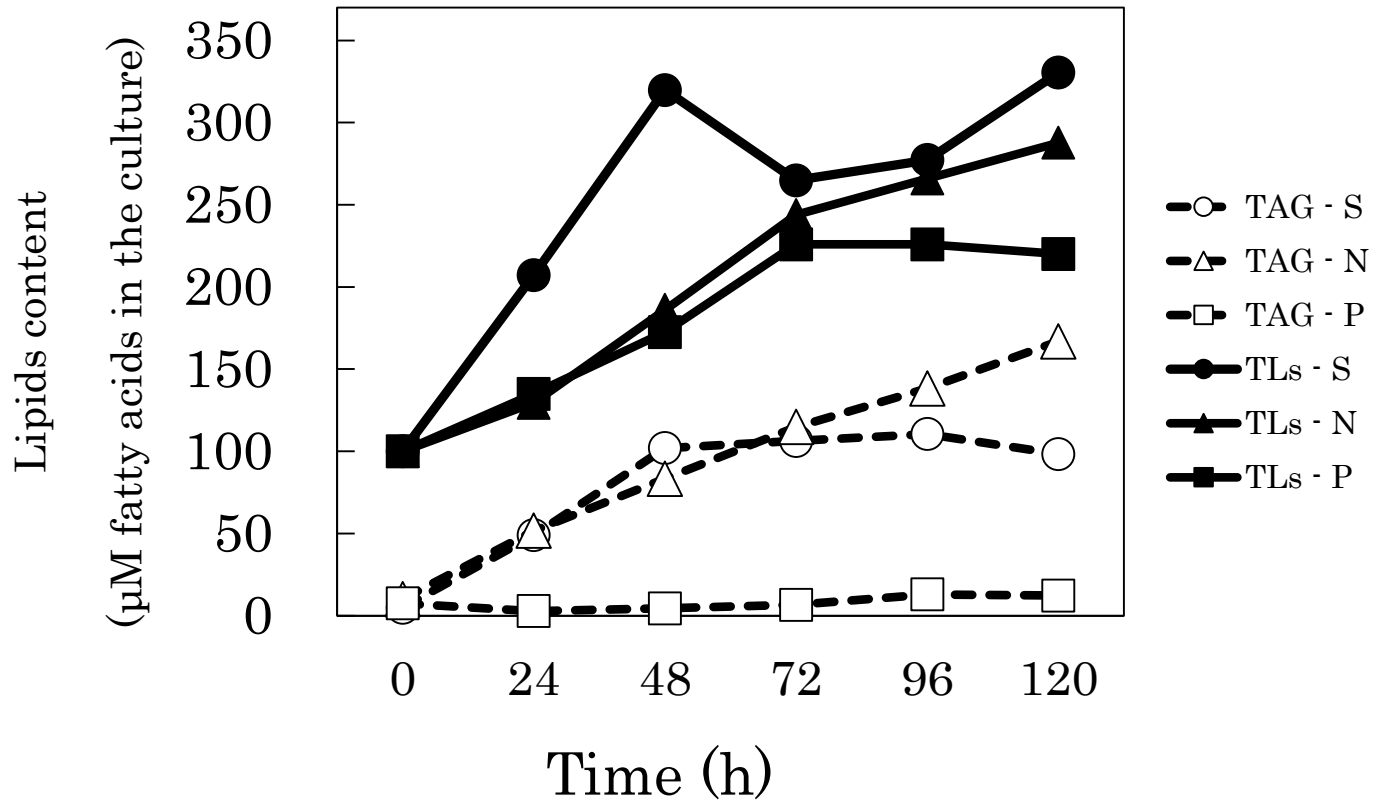


Fig. 1-6. Lipid changes in each condition. TLs amount relative to TLs in TAP-S (open circles), TAP-N (open triangles), TAP-P (open squares). Fatty acids from TAG or TLs were formed to fatty acid methyl ester (FAME), and analyzed by GC with internal standard. All values mean average ± SE determined by three independent experiments. Asterisk means that there was meaningful difference between two results ($p < 0.05$).

Table 1-1. Fatty acid composition of TAG in TAP-S, -N, -P. Fatty acids from TAG were formed to FAME, and determined by GC.

	TAG																		
	TAP-S						TAP-N						TAP-P						
	0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h	
16 : 0	35.3	32.0	30.5	29.9	29.0	29.3	35.5	33.2	32	31.1	30.2	29.7	35.9	31.2	30.1	29.4	28.8	29.1	
16 : 1(7)	1.9	4.1	3.7	3.6	3.7	3.8	2.5	2.2	2.0	2.0	1.8	1.9	2.3	1.8	1.9	1.8	1.8	1.8	
16 : 1(3t)	0.1	0.1	0.3	0.3	0.3	0.3	-	0.2	0.2	0.3	0.2	0.3	-	0.2	0.3	0.5	0.3	0.4	
16 : 2(7,10)	0.3	1.8	1.8	1.8	1.7	1.6	2.7	2.1	1.8	1.8	1.7	1.9	2.9	2.4	1.9	1.9	1.7	1.7	
16 : 3(4,7,10)	0.1	0.5	0.6	0.7	0.7	0.8	0.3	0.8	1.0	1.1	1.0	1.0	-	1.2	1.2	1.4	1.1	1.1	
16 : 3(7,10,13)	0.3	1.3	1.5	1.7	2.0	2.2	1.0	1.6	2.1	2.7	2.9	3.1	1.2	2.0	2.5	3.1	3.0	3.2	
16 : 4(4,7,10,13)	0.7	4.3	4.5	5.0	5.7	6.5	2.9	4.3	5.1	5.6	6.3	6.5	3.1	5.5	6.6	6.8	7.2	7.7	
18 : 0	3.4	3.5	3.8	3.6	3.3	3.6	4.9	3.5	4.1	4.1	4.0	4.0	5.9	3.9	4.1	3.7	3.8	4.0	
18 : 1(9)	13.9	18.6	18.1	16.1	14.0	11.7	14.1	15.1	12.6	11.0	10.1	10.0	11.9	12.2	11.2	10.0	9.2	8.6	
18 : 1(11)	8.3	6.7	7.0	7.0	6.8	6.2	7.2	7.2	7.2	7.4	7.4	7.3	9.3	8.0	7.5	7.7	7.9	7.6	
18 : 2(9,12)	15.2	12.4	12.5	12.8	12.9	12.6	13.6	14.4	12.9	11.8	11.5	11.3	14.1	15.2	12.1	10.9	10.8	10.2	
18 : 3(5,9,12)	11.0	7.0	7.4	8.0	8.6	8.8	7.5	7.2	8.1	8.2	8.5	8.3	5.2	6.8	7.6	7.7	8.1	8.0	
18 : 3(9,12,15)	9.0	7.0	7.3	8.4	9.8	11.2	8.0	7.5	9.7	11.3	12.7	13.2	8.1	9.1	11.7	13.1	14.2	14.7	
18 : 4(5,9,12,15)	0.5	1.1	1.0	1.2	1.5	1.5	1.3	0.9	1.1	1.5	1.6	1.6	0.0	0.8	1.1	1.9	2.1	1.9	
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	**(mol%)**

* Not detected

Three independent experiments were shown as average

table 1-2. Fatty acid composition of TLs in TAP-S, -N, -P. Fatty acids from TLs were formed to FAME, and determined by GC.

	TLs																		
	TAP-S						TAP-N						TAP-P						
	0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h	
16:0	20.2	24.5	27.0	26.0	25.8	25.5	23.2	28.2	29.4	30.4	30.7	30.7	23.0	27.1	28.2	28.2	29.4	29.6	
16:1(7)	1.9	2.6	2.8	3.0	3.2	3.4	2.0	1.8	1.7	1.7	1.7	1.9	1.4	1.7	2.0	1.7	1.8	1.8	
16:1(3t)	1.0	1.2	0.8	1.1	1.1	1.2	0.7	0.3	0.3	0.9	0.2	0.3	1.0	-*	0.4	1.8	0.3	0.4	
16:2(7,10)	2.9	2.2	2.0	2.1	1.7	1.7	3.2	2.2	1.5	1.3	1.5	1.7	2.9	1.9	1.6	1.3	1.6	1.4	
16:3(4,7,10)	1.7	1.2	1.0	1.1	1.0	1.0	1.8	1.6	1.1	1.4	1.0	0.9	2.1	1.6	1.1	1.0	1.1	0.9	
16:3(7,10,13)	2.8	2.0	2.3	2.3	2.3	2.3	3.1	2.2	2.4	4.0	2.7	2.6	2.7	2.4	2.4	2.5	2.6	2.6	
16:4(4,7,10,13)	11.8	9.2	7.9	8.2	7.7	8.0	10.6	8.3	7.0	6.7	6.2	6.1	10.8	9.1	7.1	6.9	6.9	6.8	
18:0	3.4	3.5	4.0	3.7	3.7	3.9	4.3	5.7	5.9	6.2	5.3	4.8	5.2	7.2	8.3	7.3	5.7	5.2	
18:1(9)	5.8	9.7	10.3	9.7	8.7	7.6	5.8	7.7	8.0	7.5	7.7	8.0	3.5	5.5	7.6	7.4	7.3	7.1	
18:1(11)	3.3	4.6	5.5	5.6	5.8	5.8	3.1	4.2	5.5	6.9	6.9	7.0	3.6	4.1	5.6	6.4	7.9	7.5	
18:2(9,12)	15.5	14.1	12.9	13.1	12.6	12.5	15.8	13.5	11	9.9	9.9	10.5	16.3	13.2	9.3	8.9	8.7	9.2	
18:3(5,9,12)	8.5	8.3	8.4	8.7	9.3	9.7	8.1	8.3	9.4	11.7	9.6	9.7	8.5	8.6	8.3	8.7	9.2	9.8	
18:3(9,12,15)	19.9	15.8	14.8	15.1	15.9	16.3	17.1	15.1	15.3	10.2	14.7	14.3	18.0	16.5	15.8	15.5	15.6	15.6	
18:4(5,9,12,15)	1.4	1.4	1.4	1.1	1.2	1.2	1.2	1.0	1.7	1.6	1.9	1.8	0.9	1.1	2.3	2.1	1.8	2.2	
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	**(mol%)**

* Not detected

Three independent experiments were shown as average

Table 1-3. Fatty acid composition of FFA in TAP-S, -N, -P. Free fatty acids from cell were formed to FAME, and determined by GC.

	FFA																		
	TAP-S						TAP-N						TAP-P						
	0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h	
16:0	21.2	25.8	26.4	26.1	26.6	31.1	29.3	32.7	29.5	33.2	38.2	35.8	40.3	36.4	31.0	35.2	39.1	34.9	
16:1(7)	1.3	1.2	1.9	2.0	2.0	2.3	3.6	-	1.9	2.5	2.6	-	7.2	-	2.0	2.4	5.1	-	
16:1(3t)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
16:2(7,10)	3.8	-	-	-	5.9	2.8	-	-	-	-	-	-	-	-	-	-	-	-	
16:3(4,7,10)	-	-	-	0.5	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-	
16:3(7,10,13)	-	-	-	-	-	0.8	-	-	-	-	-	-	-	-	-	-	-	-	
16:4(4,7,10,13)	1.7	-	-	-	-	2.2	-	-	-	-	-	-	-	-	-	-	-	-	
18:0	19.0	33.8	35.0	25.9	24.2	28.9	25.8	38.6	31.2	31	29.4	31.4	37.2	56.1	55.1	53.7	43.0	47.0	
18:1(9)	4.8	4.9	4.8	6.0	5.3	6.6	7.0	5.7	4.9	4.4	3.6	4.3	10.2	3.8	4.6	4.5	2.8	3.2	
18:1(11)	2.6	1.2	2.2	2.8	3.1	3.9	0.8	0.9	1.7	1.3	2	2.8	-	-	-	-	-	2.0	
18:2(9,12)	14.3	10.8	8.9	9.0	9.1	11.0	10.1	7.4	6.8	5.4	5.0	6.0	-	1.6	2.1	-	2.2	2.3	
18:3(5,9,12)	5.2	-	0.6	0.8	2.3	3.7	1.7	1.0	2.5	1.0	1.8	2.9	-	-	-	-	-	1.7	
18:3(9,12,15)	22.4	21.2	18.5	23.6	23.3	12.7	22.9	12.9	21.6	22.9	19.0	17.6	5.1	2.1	5.2	4.3	7.8	8.9	
18:4(5,9,12,15)	3.6	4.0	5.3	4.7	6.4	2.6	-	1.1	-	-	-	-	-	-	-	-	-	-	
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	**(mol%)**

* Not detected

Three independent experiments were shown as average

Table 1-4. Characteristics of FA in 16 cabon fatty acids (C16) and 18 carbon fatty acids (C18) ratio, and degrees of Unsaturation .

		FFA						TAG						TLs					
		0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h
C16/C18	TAP-S	0.28	0.36	0.37	0.39	0.47	0.57	0.63	0.78	0.75	0.75	0.76	0.80	0.73	0.75	0.76	0.77	0.75	0.76
	TAP-N	0.48	0.48	0.46	0.54	0.67	0.55	0.79	0.80	0.79	0.81	0.79	0.80	0.81	0.80	0.76	0.86	0.79	0.79
	TAP-P	0.90	0.57	0.49	0.60	0.79	0.54	0.83	0.79	0.80	0.82	0.79	0.82	0.78	0.78	0.75	0.96	0.78	0.77
Degrees of Unsaturation	TAP-S	1.45	0.96	0.89	1.09	1.24	1.04	1.20	1.23	1.27	1.34	1.42	1.47	1.96	1.71	1.62	1.65	1.65	1.67
	TAP-N	1.05	0.64	0.94	0.91	0.81	0.81	1.20	1.27	1.36	1.42	1.48	1.50	1.84	1.61	1.55	1.50	1.50	1.50
	TAP-P	0.33	0.13	0.26	0.20	0.36	0.42	1.13	1.37	1.46	1.51	1.55	1.56	1.86	1.66	1.51	1.50	1.53	1.54

Three independent experiments were shown as average

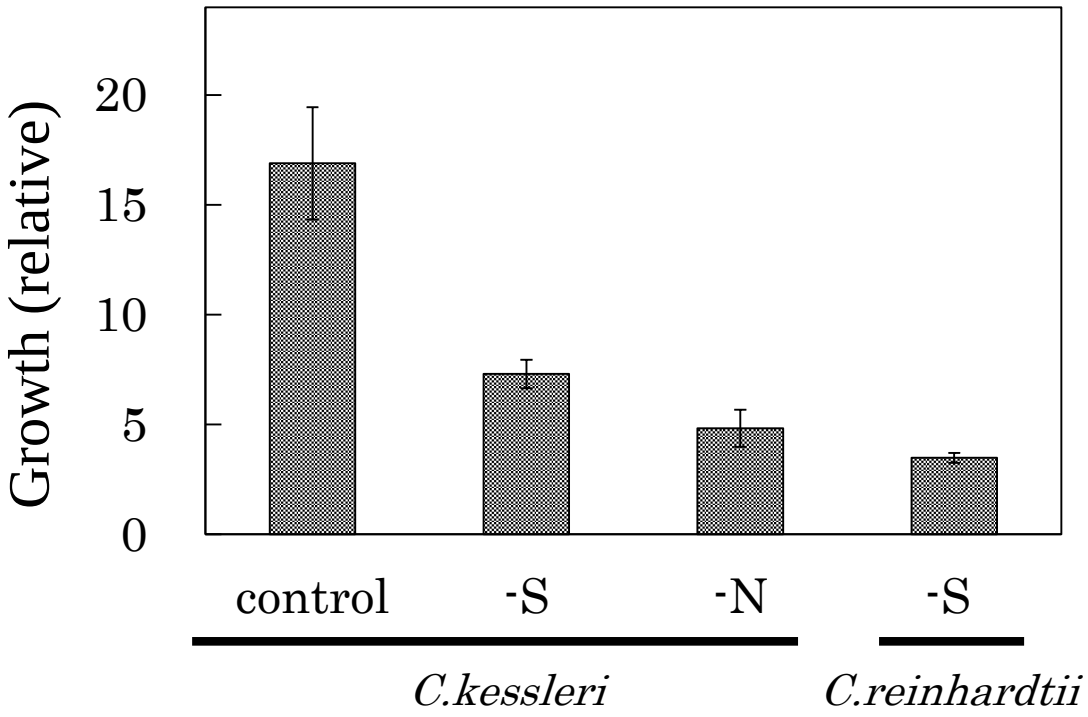


Fig. 1-7. Growth rates in 3/10HSM -S, and -N condition (after 120h). Cells were cultured with bubbling air. A_{730} was measured and set initial value to 1. Three experiments were shown as average ± SE.

C.kessleri

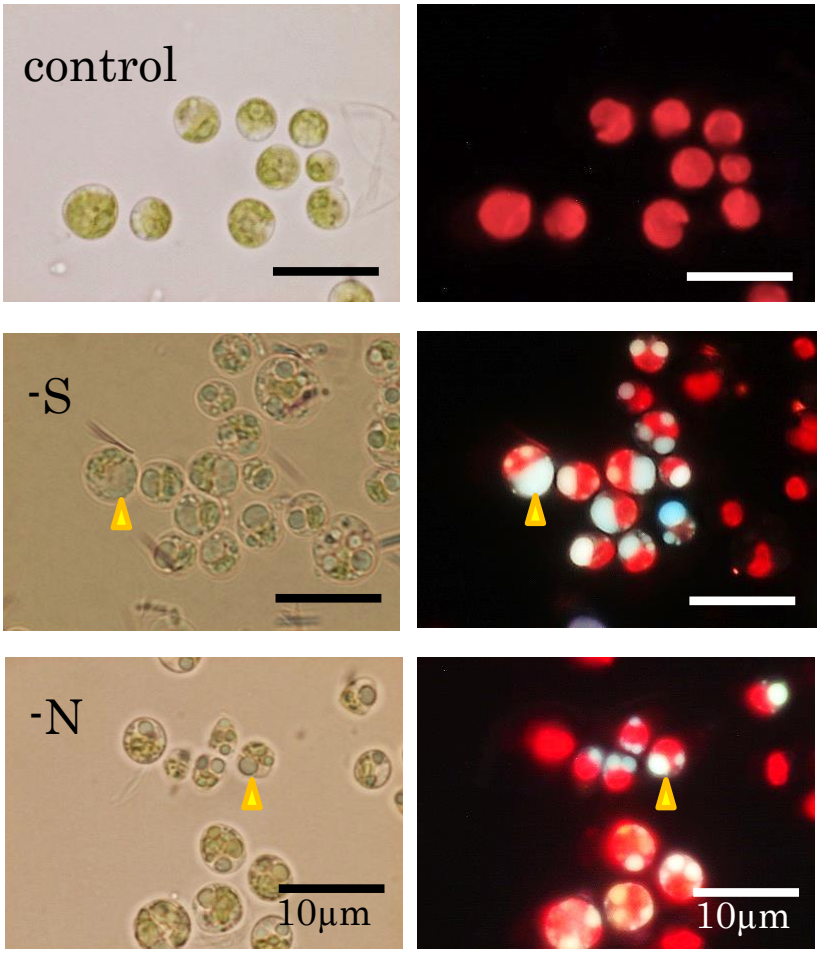


Fig. 1-8. *C.kessleri* had LDs (yellow triangles) in -S and -N (96h). Cells were cultured with bubbling air. Nile Red staining was performed under 37 °C for 20 minutes with cell.

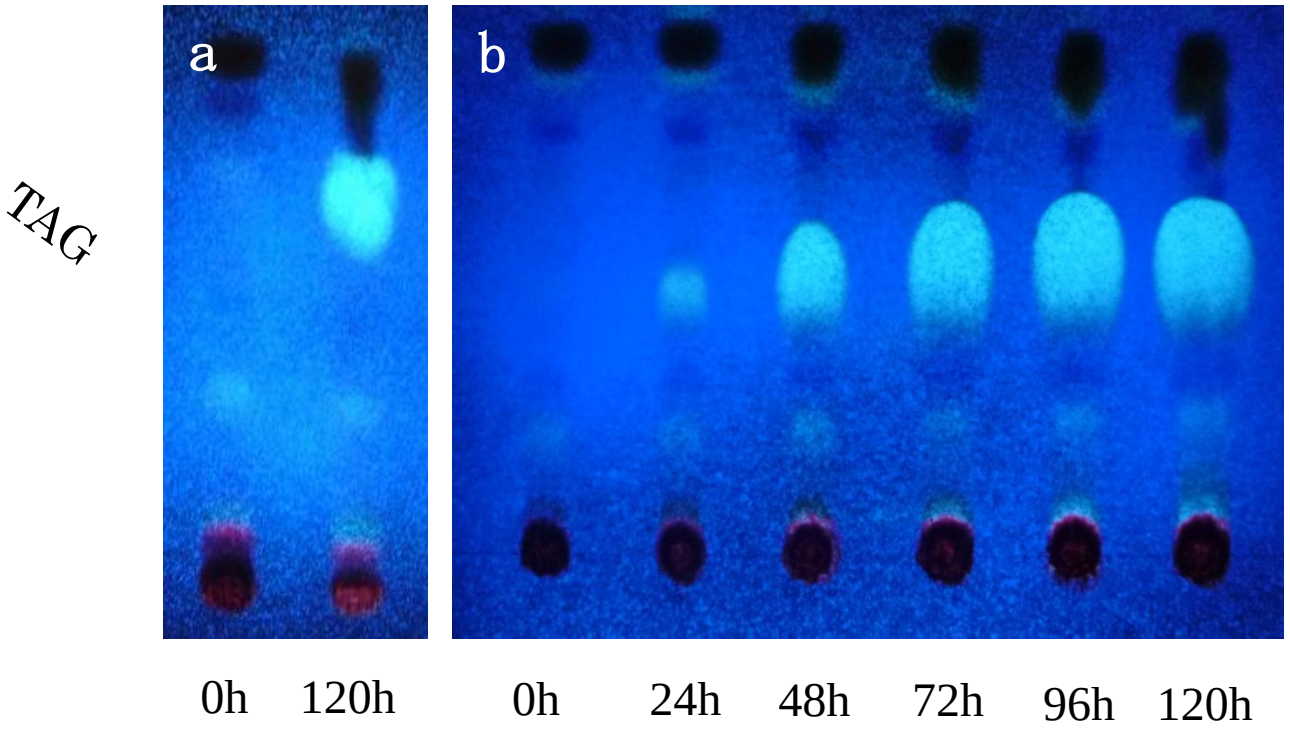


Fig. 1-9. Separating neutral lipids derived from photo-autotrophic (PA) cell by TLC (a). Lipid contents in mixotrophic (MX) or PA sulfur deficient culture, and TAP-N (b). Fatty acids from TAG or TLs were formed to FAME, and analyzed by GC with internal standard. All values mean average ± SE determined by three independent experiments.

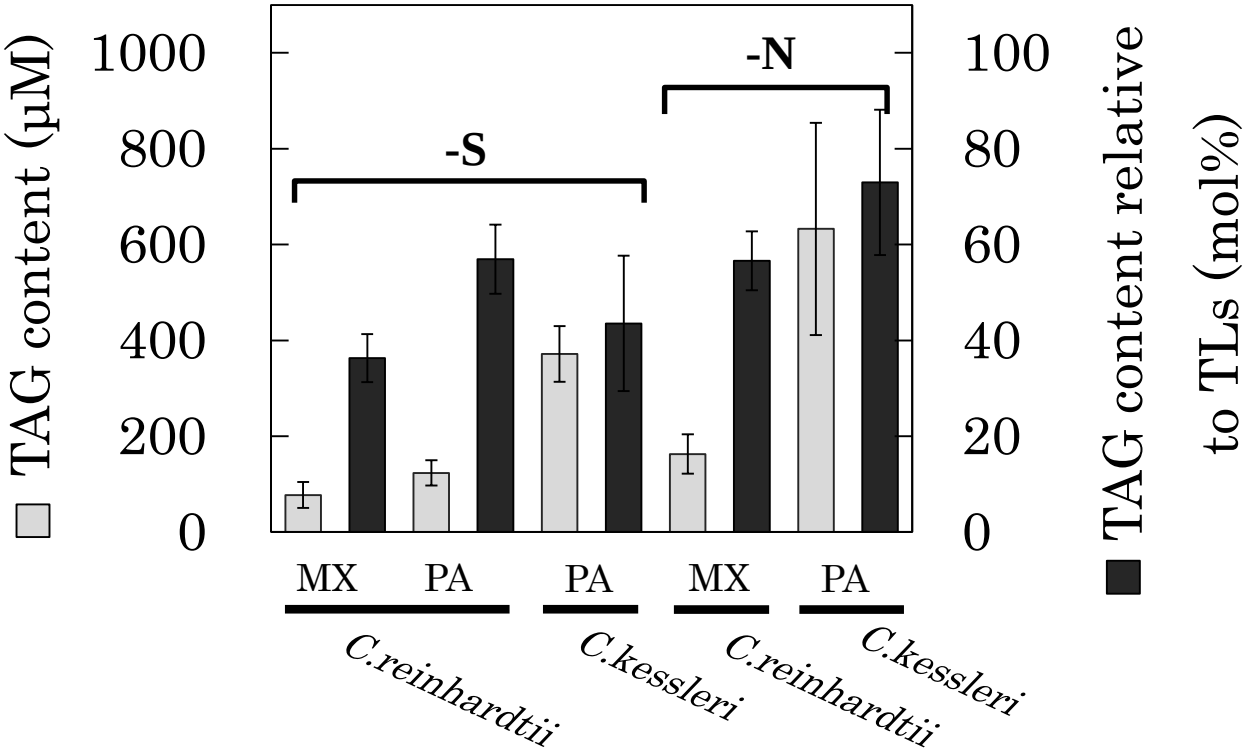


Fig. 1-10. Lipid content under various conditions in *C. reinhardtii* and *C. kessleri* at 120h. Lipid content is calculated in FA base. Photo-autotrophic (PA). Mixotrophic (MX). Values are averages ± SE for three independent experiments

Table 1-5. Fatty acid composition in *C.reinhardtii* under PA-S conditions.

	0h TLs	120h TLs	120h TAG	
16：0	21.64	29.38	27.14	
16：1(7)	2.97	5.95	6.51	
16：1(3t)	1.87	0.67	0.27	
16：2(7,10)	3.04	1.58	1.65	
16：3(4,7,10)	2.32	0.53	0.54	
16：3(7,10,13)	2.77	2.99	3.48	
16：4(4,7,10,13)	13.52	4.60	4.36	
18：0	5.90	4.83	3.10	
18：1(9)	4.00	12.32	15.93	
18：1(11)	3.96	7.45	7.96	
18：2(9,12)	10.30	10.40	11.11	
18：3(5,9,12)	6.99	8.17	7.53	
18：3(9,12,15)	19.50	9.76	9.10	
18：4(5,9,12,15)	1.19	1.37	1.33	**(mol%)**

Three independent experiments were shown as average

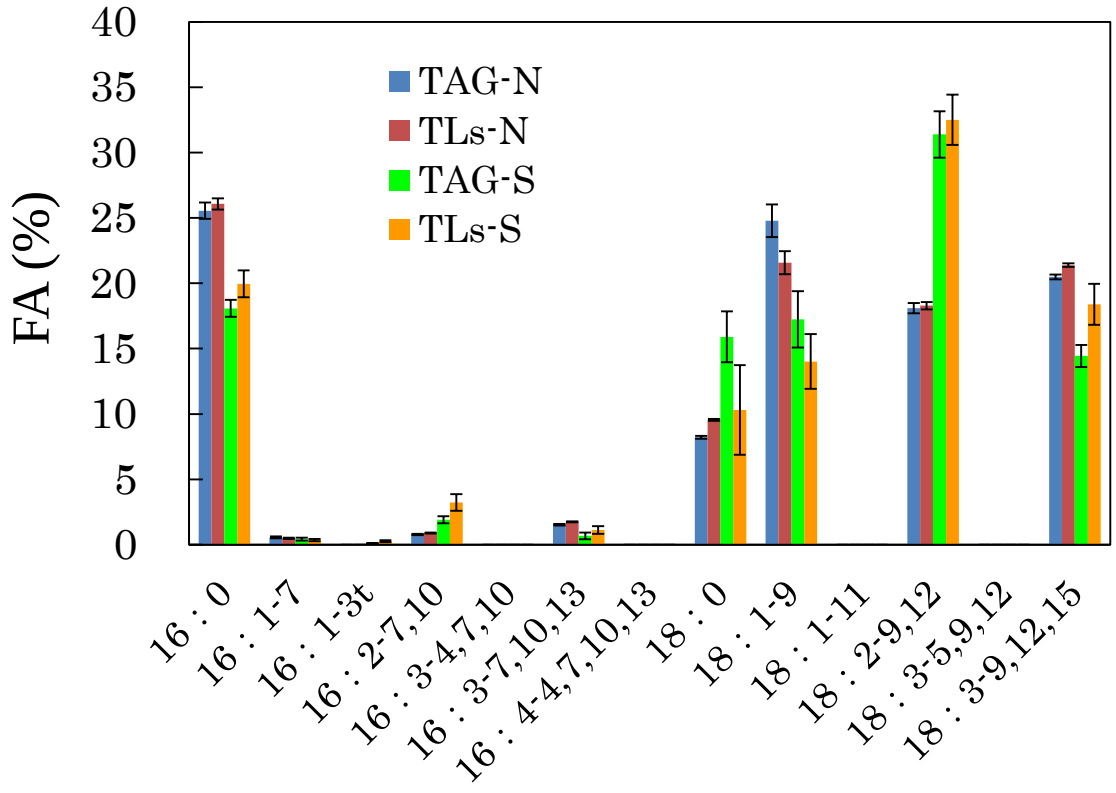


Fig. 1-11. Fatty acid (FA) composition of TAG and TLs in *C.kessleri*. Values are averages ± SE for three independent experiments

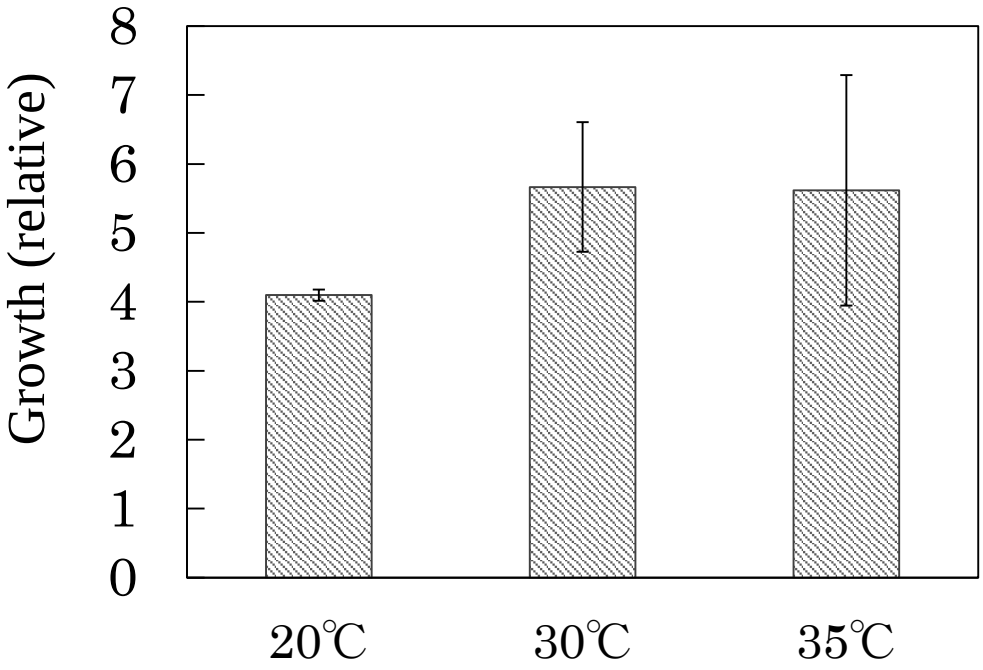


Fig. 1-12. Effect of temperature on growth rate under -S conditions in *C.kesseri* (120h). Cells were cultured at 30°C as control. Values were related on A_{730} at 0h. Three experiments were shown as average ± SE.

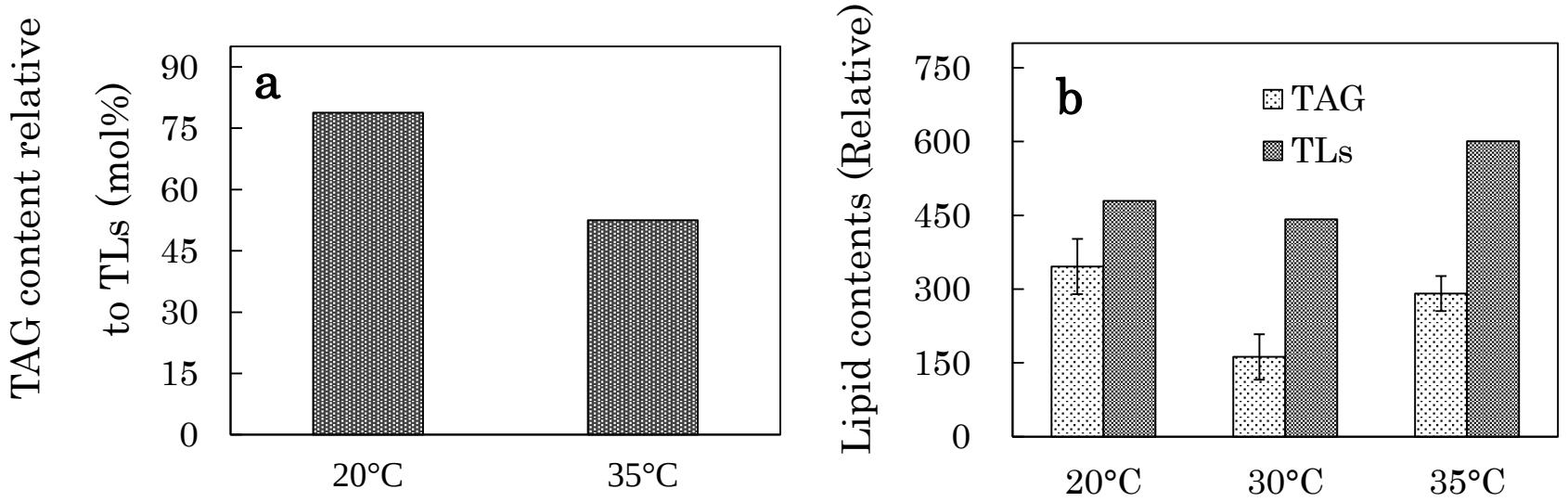


Fig. 1-13. Effect of temperature on lipid content under -S conditions in *C.kesseri* (120h). S-starved cell at 20°C highly accumulated TAG 78.8% (a) than at 30°C (Fig. 4-3, 43.5%). Value s in Fig. **b** relative to cell volume (described in fig 3-1), and were based on FA. Values are averages for two experiments except TAG in Fig. **b** (average ± SE, n=3)

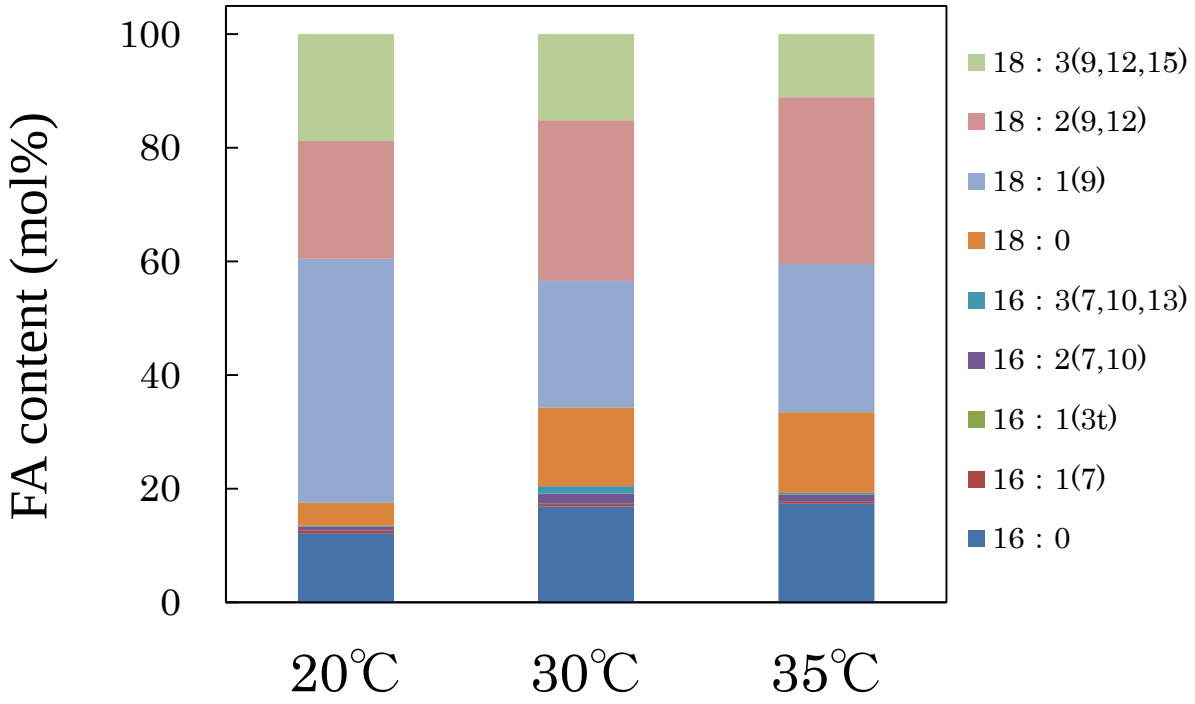


Fig. 1-14. Effect of temperature on FA composition under -S conditions in *C.kesseri* (120h). Three experiments were shown as average.

Table 1-6. Lipid characteristics by temperature. TAG was prepared from S-starved *C.kessleri* cells at 120h

	20℃	30℃	35℃
Degrees of unsatulation*	1.41	1.23	1.21
Melting point(°C)**	11.1	19.9	19.7

Values are averages *± SE<0.05, **± SE<1.50

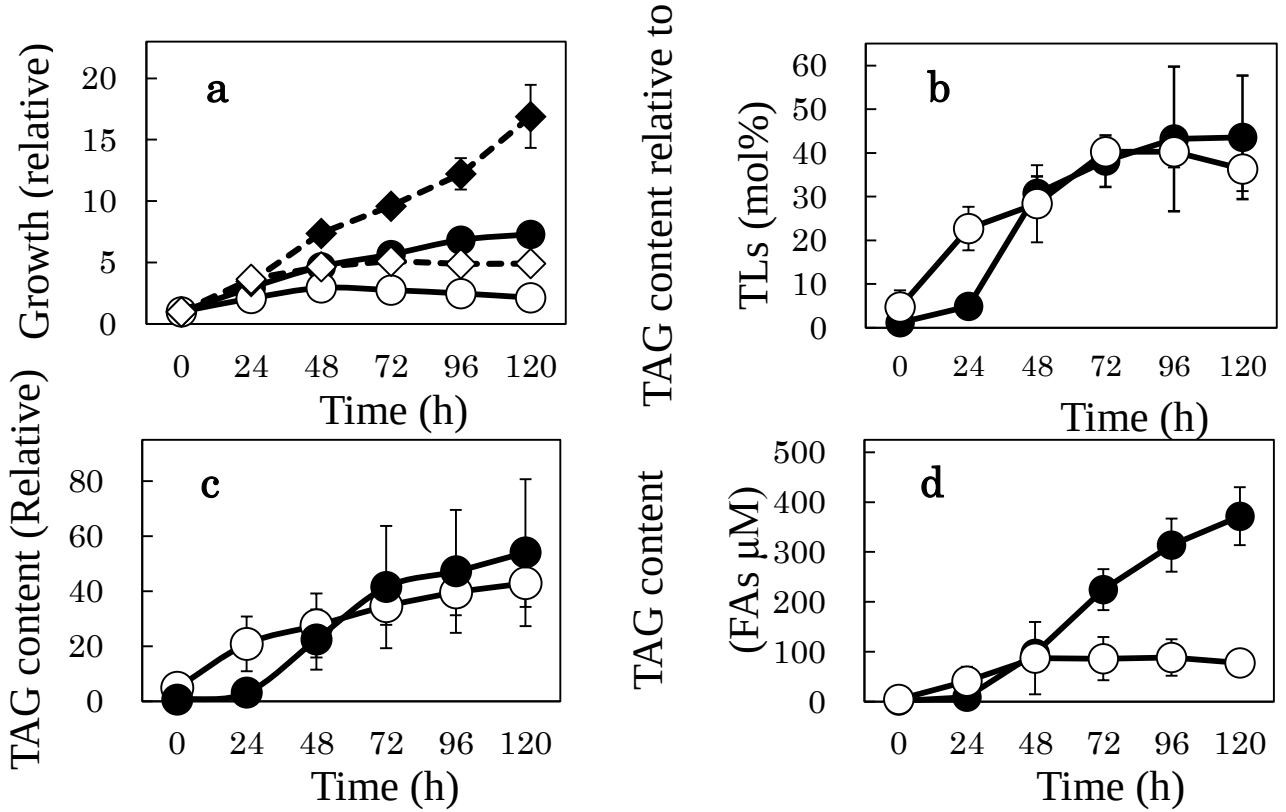


Fig. 1-15. Comparing the cells between *C. reinhardtii* (open symbols) and *C. kessleri* (closed symbols). *C. reinhardtii* cells were cultured with TAP medium (Fig. **a**, open diamonds), and 3/10 HSM was used for *C. kessrelei* (closed diamonds). Values in Fig. **c** relative to cell volume (described in Fig. 3-1), and were based on FA. TAG content relative to culture liquid is shown in fig **d**. Values are average ± SE (n=3)

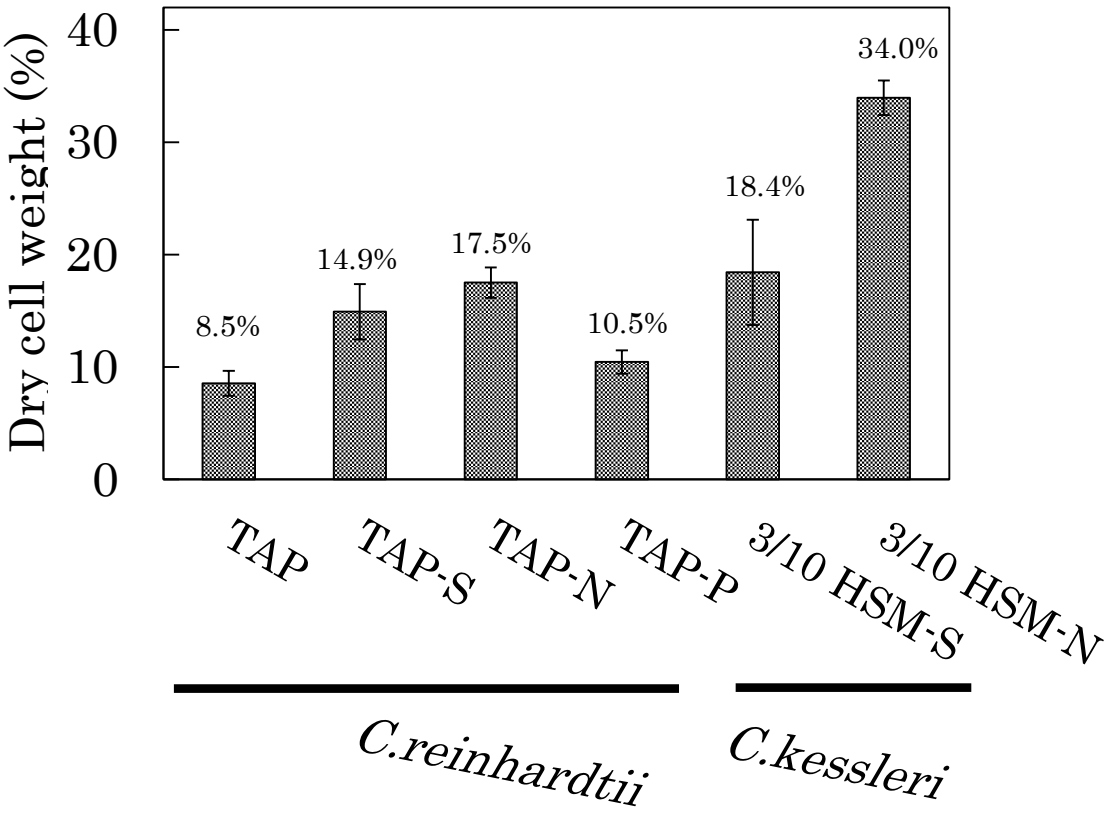


Fig. 1-16. Lipid amounts relative to dry cell weight. Total lipids were extracted as FAME from dried cell directory with *n*-hexane. CT: TAP 0h, S: TAP-S 120h, N: TAP-N 120h, P: TAP-P 120h. Values mean average ± SE determined by three independent experiments.

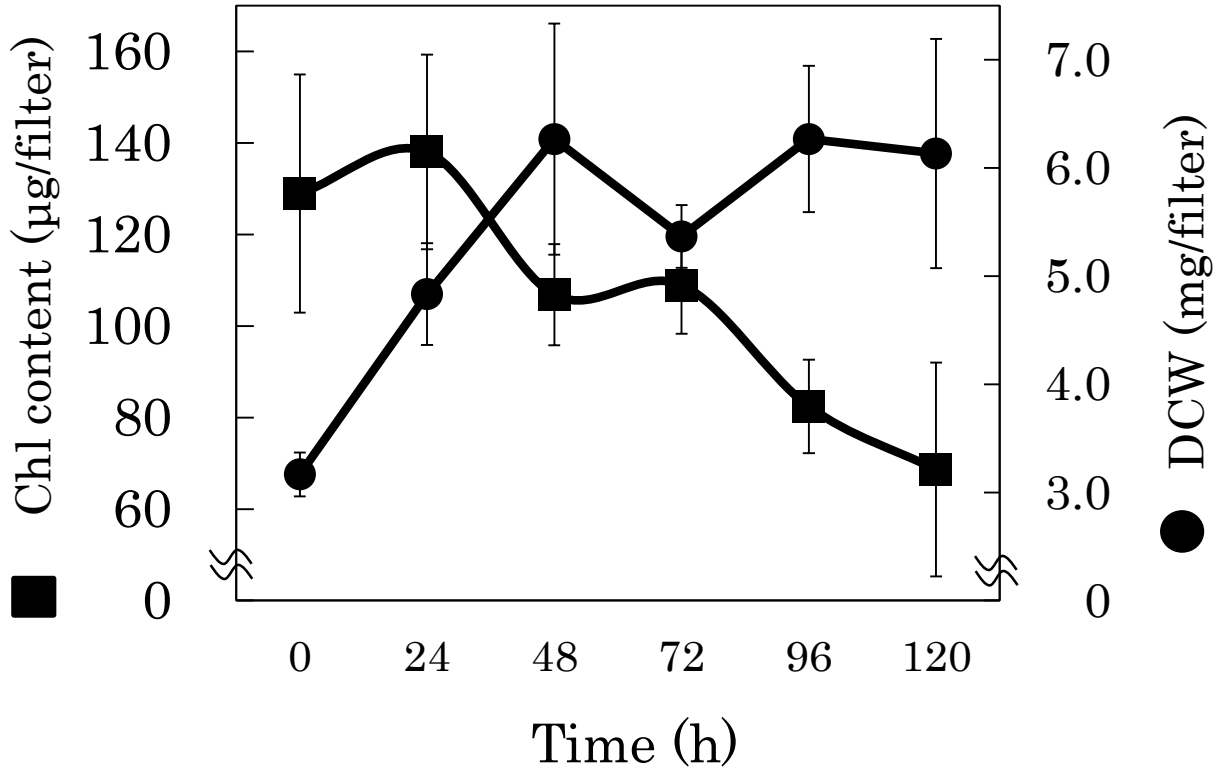


Fig. 1-17. Chlorophyll content (closed squares) and dry cell weight (DCW, closed circles) changed during regular air drying (RAD) conditions. Total chlorophyll was extracted from whole cells on a filter with methanol. DCW was calculated: Total weight – filter weight (after dried completely with drying-oven). The values are averages ± SE in three filters at same periods.

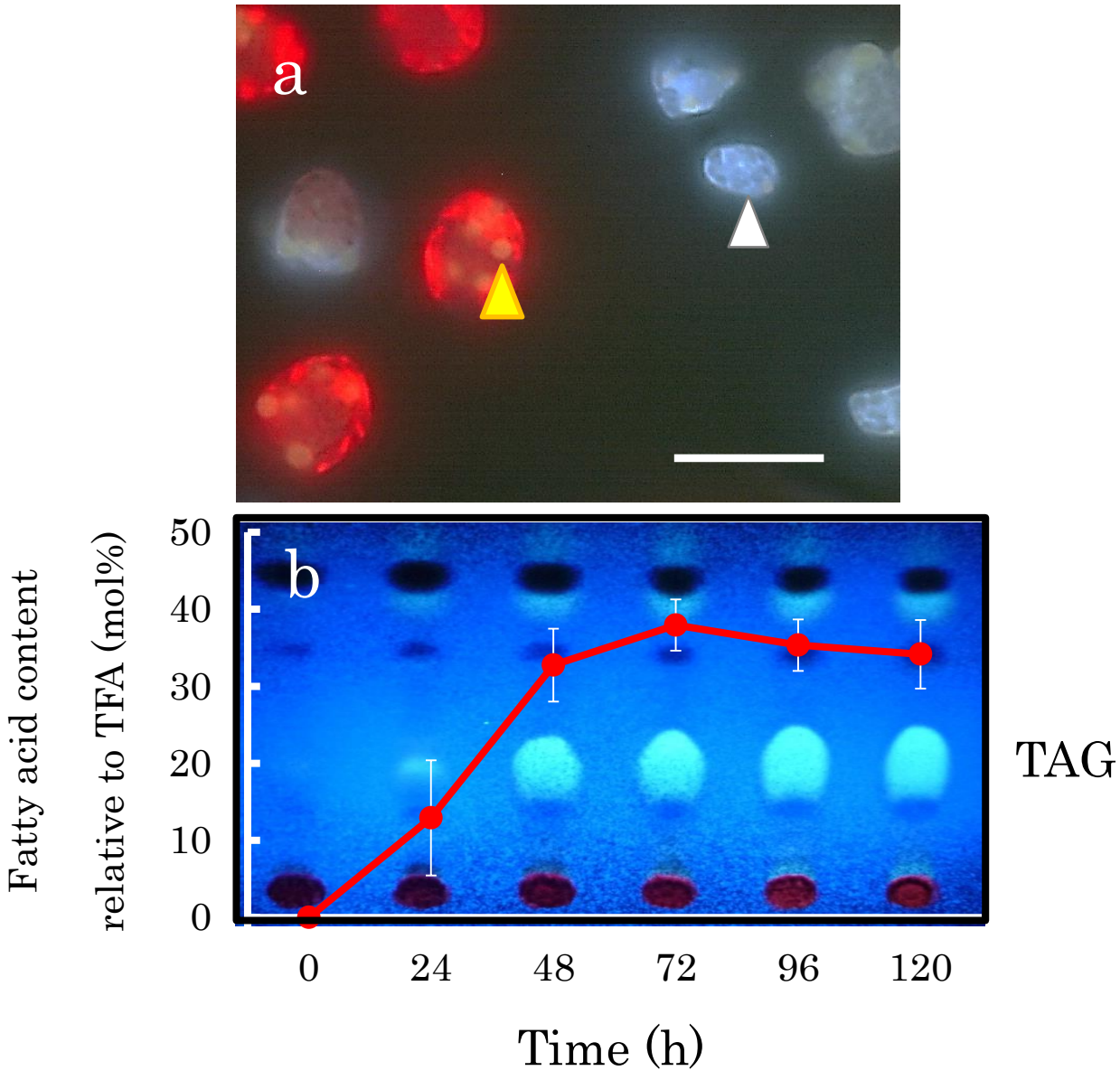


Fig. 1-18. Nile red staining of RAD cells at 96h (a). LDs were observed in some cells (yellow triangle), some cells were discolored (white triangle). Scale bar means 10μm. FA derived from TAG relative to TFA (red circles) with TLC background (b). The values are averages ± SE in three filters at same periods respectively.

Table 1-7. Fatty acid composition in *C.reinhardtii* under RAD conditions.

	TAG						NL+PL					
	0h	24h	48h	72h	96h	120h	0h	24h	48h	72h	96h	120h
16 : 0	-*	35.1	28.1	27.5	26.6	27.0	19.8	22.3	24.7	26.1	26.5	30.2
16 : 1(7)	-	6.8	5.6	4.7	4.3	3.8	4.5	4.5	4.2	4.0	3.6	3.7
16 : 1(3t)	-	-	0.1	0.2	0.2	0.1	1.5	1.5	0.9	0.6	0.5	0.5
16 : 2(7,10)	-	0.8	1.7	1.7	1.8	1.8	3.4	2.4	2.5	2.1	1.5	1.7
16 : 3(4,7,10)	-	0.5	0.7	0.8	0.9	1.0	2.3	1.7	1.4	1.4	1.2	1.3
16 : 3(7,10,13)	-	1.0	1.7	1.9	2.2	2.3	2.6	2.3	2.4	2.2	2.1	1.8
16 : 4(4,7,10,13)	-	3.6	4.6	5.2	6.0	6.4	12.2	11.1	8.9	8.7	8.1	6.8
18 : 0	-	13.8	4.4	4.3	4.3	4.6	8.9	7.8	7.1	6.1	7.4	12.7
18 : 1(9)	-	13.6	14.6	11.6	11.1	9.0	9.7	9.5	8.8	7.5	5.7	6.1
18 : 1(11)	-	8.5	13.7	14.4	13.9	14.2	2.9	4.5	7.7	9.2	9.8	9.8
18 : 2(9,12)	-	6.4	9.8	10.6	10.4	9.9	8.4	9.4	10.2	9.4	7.0	6.7
18 : 3(5,9,12)	-	4.7	6.6	7.1	7.2	7.4	5.2	5.2	5.7	6.4	7.8	5.7
18 : 3(9,12,15)	-	4.7	7.1	8.6	9.7	10.8	17.2	16.6	14.6	15.1	17.5	12.0
18 : 4(5,9,12,15)	-	0.6	1.1	1.4	1.4	1.8	1.2	1.2	0.9	1.1	1.3	1.0
	-	100	100	100	100	100	100	100	100	100	100	100

(mol%)

Three independent experiments were shown as average
* Not detected

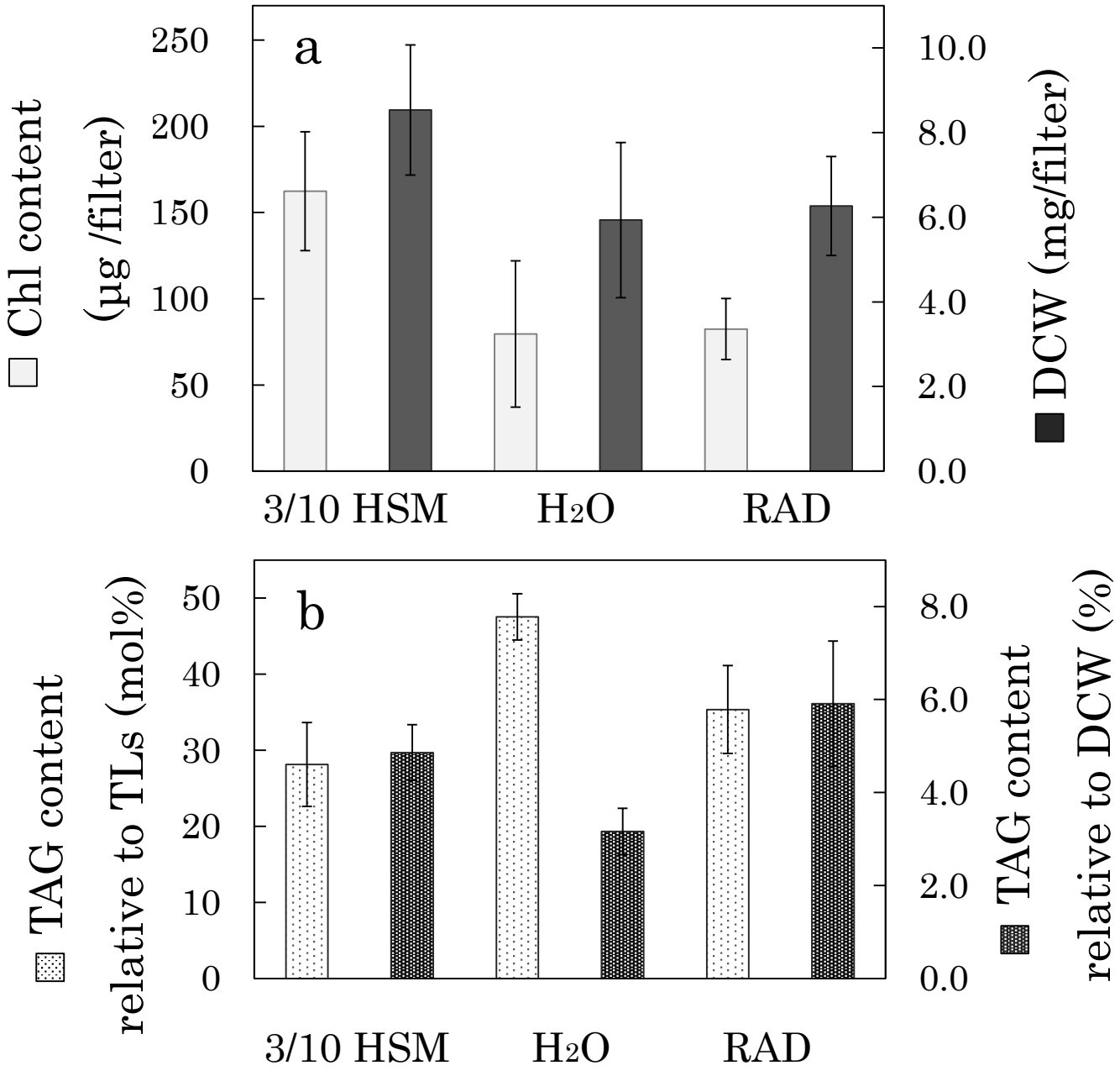


Fig. 1-19. Influences of supplying culture or H_2O on growth (a) and TAG accumulation (b) after 96h RAD conditions. Chl content (white bar), DCW (black bar), TAG content relative to TLs (white bar with dot), TAG content relative to DCW (black bar with dot). Values are averages ± SE for three filters at the same term.

1-4 Discussion

Algal TAG accumulations in nutrient deficiency were shown in this chapter. It is known that algae store TAG in stress conditions such as nitrogen deficiency, high salt etc. In addition to these conditions, it was found that sulfur-starved stress also triggered the TAG accumulation. Stressed cells tended to become large, and, the structure of chloroplast was disintegrated under -S or -N conditions (Fig. 1-3). Cell hypertrophy is also known in a SQDG deficient mutant and temperature dependent cell division mutant (2004 Imai master thesis, Brandt et al., 2012), SQDG redistributes and declines actually in S-starved cells (Sugimoto et al., 2007). Chl decreasing supports the disintegration of chloroplastic structure. Electron microscopy helps the understanding to the detail of this chloroplast redistribution. It appears that starch oppresses the grana at chloroplast in N-starved cells (Fan et al., 2011). Sulfur deficient condition also induce starch amount in *C. kessleri* and *C. reinhardtii* (Brányiková et al.,2010; Mizuno et al., 2013, Russa et al 2012), and the author confirmed, too (supplemental Fig. 1-a). LDs which contain TAG are surrounded by monolayer phospholipid with oleosin, caleosin, and steleosin that are alkali-proteins in land plant (Tzen et al., 1992, Purkrtova et al., 2008). The function of these alkali-proteins has not been definite, but oleosin and caleosin were considered that control the size or stabilization of LDs (Purkrtova et al., 2008, Chapman et al., 2012). *Chlamydomonas* have oleosin-like protein, and also functions as well as land plant. However *Chlorella* has not been reported about oleosin. The author tried to seek oleosin-like protein but there was no positive evidence in *Clollera*. This was the reason why the size of LDs in *C. kessleri* was larger than that of in *C. reinhardtii* (Fig. 1-8). Moreover, *C. reinhardtii* have LDs specific protein called major lipid droplet protein (MLDP) that is super hydrophobic protein (Neguyen et al., 2011). Recent study has developed the understanding of LD formed process, LDs are localized in cytosol mainly, but chloroplasts and endoplasmic reticulum (ER) cooperate to form LDs (Goodson et al., 2011).

Separating NLs by TLC make TAG observation clearly according to the volume of LDs. However, since LDs including TAG is synthesized generally, TLC analysis does not give an actual TAG content. Therefore quantitative analysis was performed by GC after methyl esterified FAs which were held in lipids including TAG. As the results, the maximum TAG contents relative to TLs were different as 40.3%, 56.6%, 7.7% in -S, -N, -P respectively. This result indicated that the level of TAG accumulation is dependent on the conditions which trigger TAG enhancement. In other words, TAG accumulating mechanisms are controlled and determined the lipid content by each condition. At the results of TAG content per culture medium, -N condition increased TAG amount until 120h but less increasing TLs than contrast to in -S condition (Fig. 1-6, Supplemental Fig. 1-b). It was considered that PLs synthesis more likely proceeded to cell membrane constitutions in S-starved cell than N-starved cell. Sulfur

contained lipid as SQDG and nitrogen contained lipid as DGTS are in membrane lipids. Former is minority lipid, nevertheless participates in photosynthesis as one of thylakoid membrane lipid, and supports its activity (Sato et al., 1995, Aoki et al., 2004). DGTS is abundant as about 20% in membrane lipid, and it is the most major lipid which don't compose thylakoid membranes (supplemental table. 1-a). It is also known that DGTS synthetic enzyme is contained in LDs (Neguyen et al., 2011). Thus, less increase of TLs under -N conditions than -S was explained by balance of DAG supply in the view of nutrient necessity. DGTS was able to be synthesized in -S, and DAG was used for TAG and other lipids including DGTS [also known phospholipids increasing in -S (2009 Sato master thesis)] synthesis. In the -N condition, DAG flow would incline to TAG synthesis.

TAG of *C. reinhardtiii* contained palmitic acid, oleic acid, linoleic acid (table 1-1), and besides in *C. kessleri* included stearic acid. For lack of desaturate enzyme, FA composition of *C. kessleri* was simpler than that in *C. reinhardtii*. These major FAs in algae were similar to palm (fruit) oil which is the most used in the world. Melting point was about 20°C (data not shown). Included other C16 and C18 unsaturated FFAs, FA variety was shown in green algae. Notably, TAG involved 16:1 (3t) FA which is present in PG particularly. This result suggested that TAG synthesis involved in FAs reusing. However, main FFAs were palmitic acid, stearic acid, and linolenic acid. Utilization of FAs from distributed lipids was minority, since FA composition of TAG did not increase these FAs. Degrees of unsaturation of TAG increased in each condition at 120h (table 1-4). That of TLs did not show the tendency, it was characteristic in TAG. Getting degrees of unsaturation gives fluidity to cell membrane and results of surplus energy consumption (Sugimoto et al., 2008). TAG may have a possibility to receive surplus energy, because that would not compose cell membranes.

TAP medium includes acetate as carbon source, and acetate is involved in FA synthesis as acetyl-CoA. Acetate acts extremely to the volume of LDs under N-starved conditions in starch-less mutant *C. reinhardtii* (Goodson et al., 2011), so that it is considered that adding carbon source is important to TAG accumulation. However, the author showed high TAG accumulation in PA growth (Fig. 1-10). The volume of supplied carbon source by bubbling air may be higher than use of TAP, because acetate in TAP was used rapidly and be exhausted within a day (Ramanan et al., 2010). Therefore, the author concluded that carbon acquisition in cell was important but not existence of acetate or MX growth condtions. In addition to, the used starch-less mutants also known as high TAG accumulation under -N condition (Wang et al., 2009, Li et al., 2010a). These cells would show differences easily in TAG accumulation.

Temperature influenced FA composition of *C. kessleri* (Fig. 1-14), accompanied with increase of TAG content (Fig. 1-13b). At low temperature 20°C, TLs hardly increased but TAG increased, so that TAG volume relative to TLs showed high status as 78.8% (Fig. 1-13a). Furthermore,

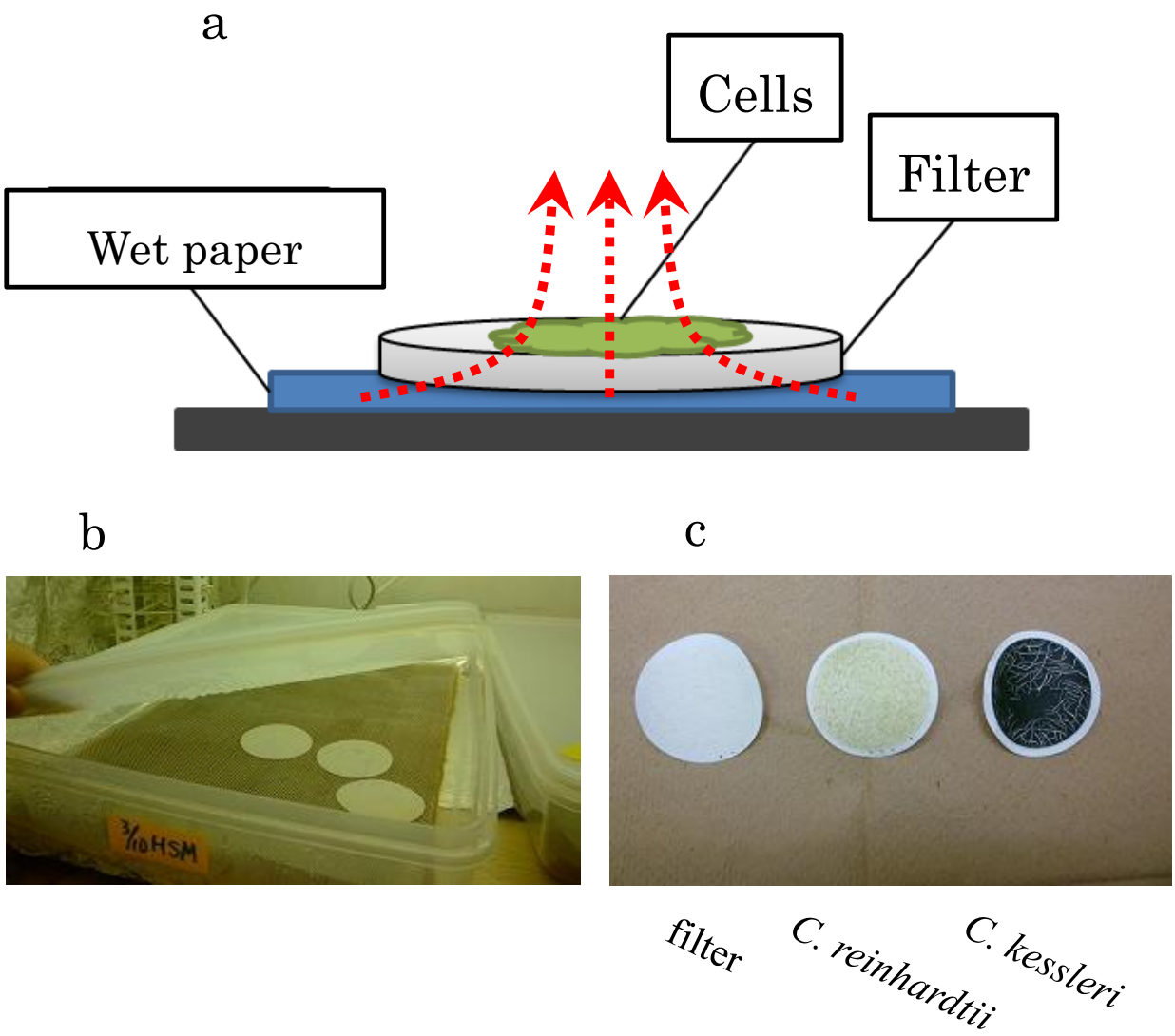
growth rate at 20°C was less than in usual condition as 30°C. Membrane lipids which are necessary for cell division wouldn't be undergone. Changing FA composition by temperature is useful for manipulating lipid quality without gene modifying. In the aspect of industrial application, poly-unsaturated FAs are expected compound with profits. It is also known that concentration of CO_2 affect FA composition (Tsuzuki et al., 1990). Meanwhile, the author examined same experiments in *C. reinhardtii*, but that cells showed high sensitivity to the temperature. Therefore cells were extinct at 35°C and little growth at 20°C (data not shown). However recent study reported FA changing in *C .reinhardtii* by temperature (Yao et al., 2012).

There were no differences in TAG accumulating content of *C .reinhardtii* and *C. kessleri* under -S condition (Fig. 1-15b, c). However *C. kessleri* grew 3.5-fold as high as *C. reinhardtii* under -S condition, and it allowed high TAG produce ultimately (Fig. 1-15a, d). Thus, to produce TAG valuably, it is important that cell tolerance to environmental loads.

Applied technology using algae is limited by high cost such as light source, harvest, and water etc. Algae differ from large plants in that algae have problems to maintain and to remove water. Then, our laboratory developed culture methods using on cloth or paper with minimum medium named regular air drying (RAD) condition. The author examined the method in *C. reinhardtii* to understand TAG accumulation. *C. kessleri* also cultured and reported by Shiratake master thesis in 2012. As a result, *C. reinhardtii* was able to glow on glass-fiber filter as 6.0-fold at 48h in DCW. However, it was found that about 40-50% cell were died by microscopy observation (Fig. 1-18a). Comparing with *C. kessleri* (supplemental Fig.1-d), *C. reinhardtii* had high sensitivity to RAD stress. Although cell dying, TAG accumulating was also found under RAD conditions, and showed 37.9% at 72h (Fig. 1-18b). Thus, it is found that is possible for *C. reinhardtii* to grow under RAD conditions, and it leads TAG accumulation. However, *C. reinhardtii* could not exhibit TAG accumulation completely because of high intolerable stress. Actually, supplying culture medium from bottom permitted DCW increasing (Fig. 1-19a). It is difficult to figure out clearly that what RAD stress is. However, there were a few hints to help understanding. At first, RAD condition appeared to be complex stress with the both of dehydration and nutrient deficiency. Limiting water leads inhibition to essential metabolism in cells such as hydrolysis, or cell density stress by shortage of habitant space. Secondly, Fig. 1-19b indicated that nutrients are limiting factor to glow on filter but, it showed TAG accumulation. On the other hand, supplying H_2O could enhance TAG content per TLs. These results suggest that limiting water affected cell metabolism not density stress. Moreover, dehydration stress affects TAG accumulation actually, but dehydration stress would be severe for *C. reinhardtii*. Thus RAD stress in *C. reinhardtii* was concluded that TAG accumulation caused by dehydration and nutrient-deficiency, which limited by cell viability. Aspects of applied technology, showing algal cultivating on filter have great meaning. This cultivating

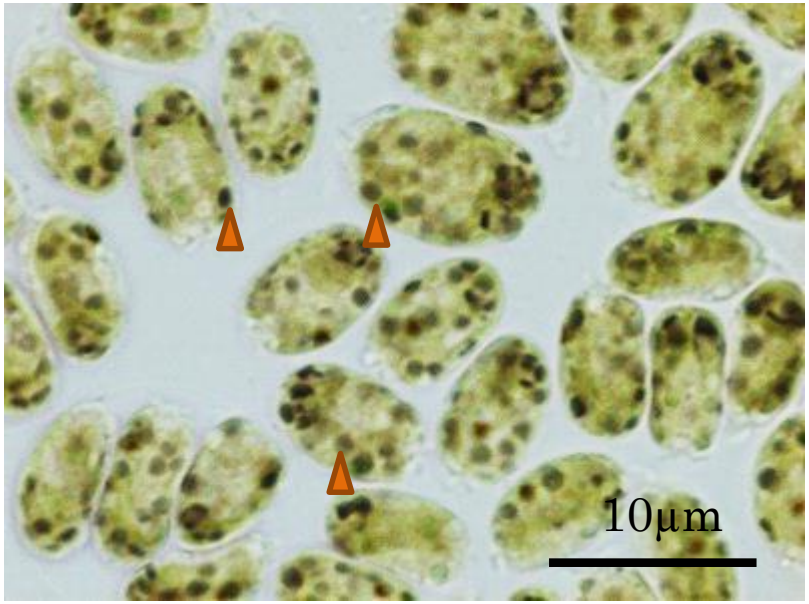
method resolves water control such as temperature and necessity of strong container, and complex harvesting process.

In the above, the author found TAG enhancement by sulfur-starved condition and indicated that stress-dependent mechanisms control TAG accumulation. Sulfur and nitrogen are essential for protein synthesis. Disorder of protein metabolism may have a relationship to TAG accumulation.

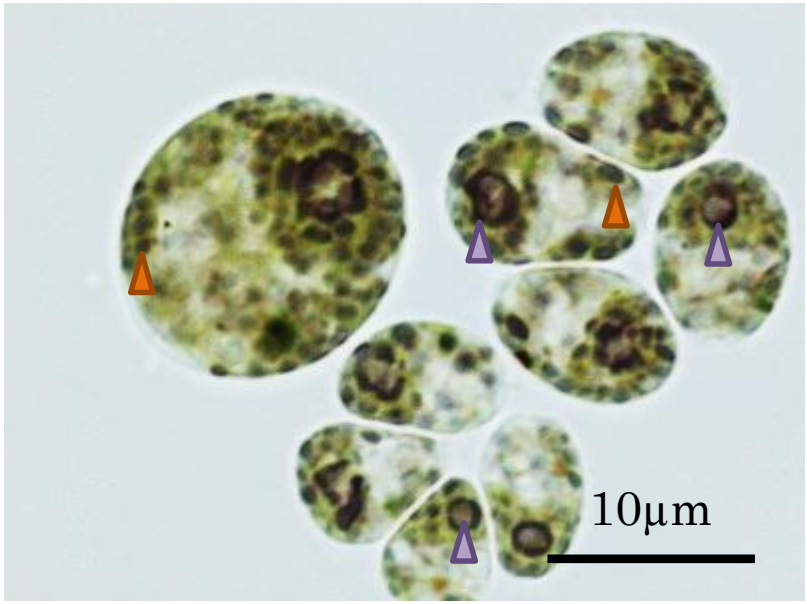


Supplement Fig. 1-a. Model of RAD conditions (a), actual cultivating situation (b), and harvest filters after dried (c). Keep the humidity more than 99% in the box (see drip on lid in Fig. 1-b), and water source is limited only from wet paper (except supplying H_2O).

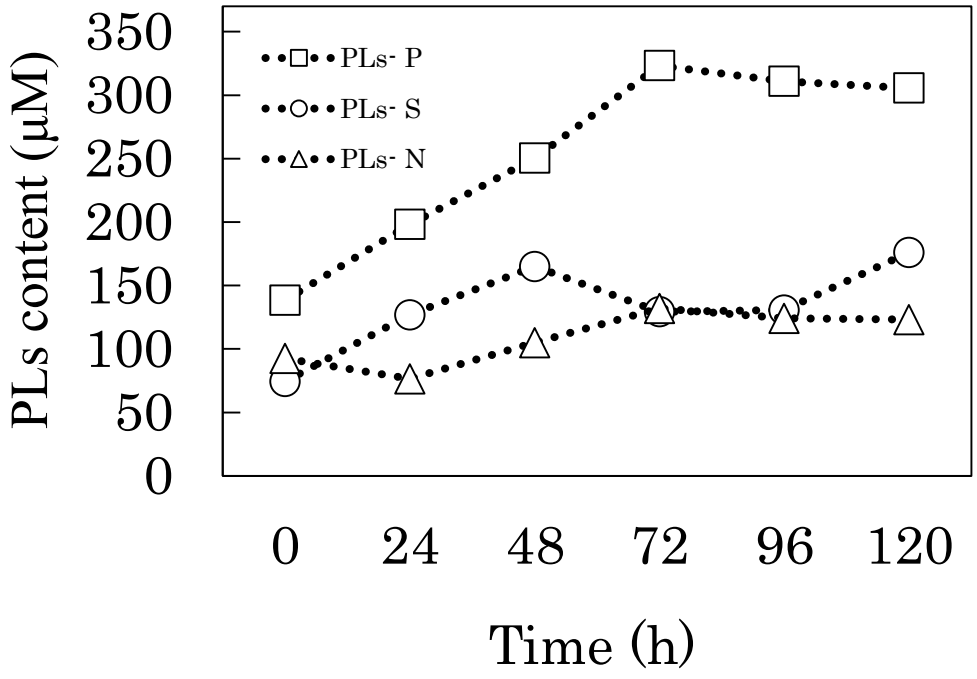
TAP



TAP-S



Supplement Fig. 1-b. Starch staining in *C.reinhardtii* at 24h. Stroma starchs were observed in both of TAP and TAP-S (brown triangles). Pyrenoid starchs were developed especially in TAP-S (purple triangles).



Supplemental Fig. 1-c. Polar lipids changes under nutrient deprived conditions. -S: open circles, -N: open triangles, -P: open squares. Values are averages for three experiments.

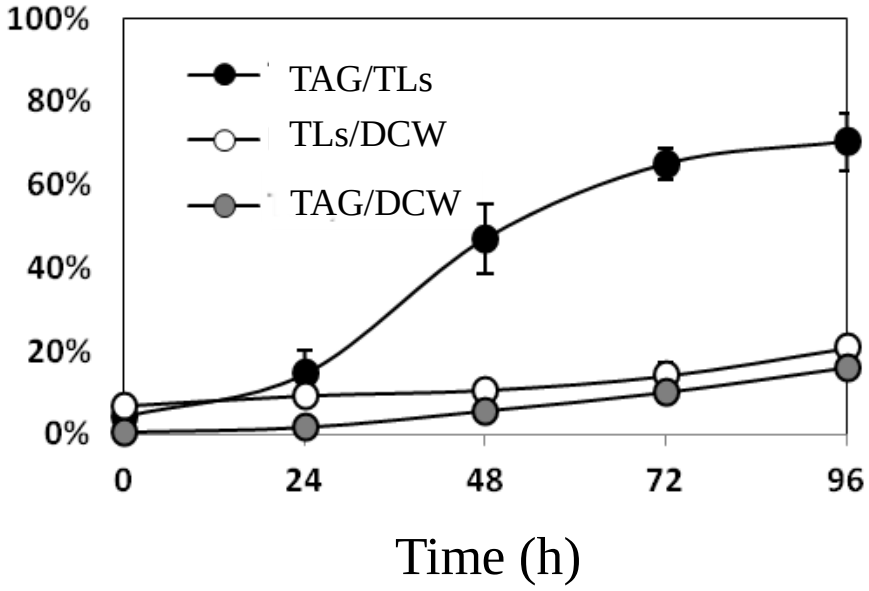


Fig. 1-d. Lipid content in *C. kessleri* under RAD conditions (Shiratake and Sato et al., 2013). Total lipids (TLs), dry cell weight (DCW). Values are averages ± SE for three filters at the same term.

(Shiratake and Sato et al., 2013)

Table1-a. Fatty acid composition of polar lipids

cc125	MGDG	DGDG	SQDG	PG	DGTS	PE	PI
16:0	1.59±<0.0	39.4±4.0	83.3±4.4	42.5±3.2	39.3±1.5	6.75±1.5	55.7±3.6
16:1(7)	0.99±0.5	-	-	-	-	0.45±0.8	-
16 : 1(3t)	-	-	-	15.8±5.3	-	-	-
16:2(7,10)	6.56±2.4	5.24±0.5	-	3.13±0.6	0.65±0.1	-	-
16:3(4,7,10)	3.28±0.3	0.25±0.4	-	-	-	-	-
16:3(7,10,13)	5.50±1.2	2.89±2.6	-	-	0.55±0.1	-	-
16:4(4,7,10,13)	33.3±2.0	4.98±4.0	-	1.97±2.3	0.79	1.61±2.8	-
18:0	0.25±0.2	0.60±0.3	1.80±0.7	2.40±1.3	0.95±0.2	29.7±2.2	6.58±1.6
18:1(9)	2.01±1.1	6.01±2.8	1.65±0.5	3.01±2.7	4.76±2.9	2.27±0.7	35.6±2.6
18:1(11)	0.20±0.3	0.82±1.4	1.66±0.5	7.79±2.3	1.74±1.5	6.02±0.8	-
18:2(9,12)	12.0±2.6	19.7±2.3	4.89±0.8	15.4±0.4	18.2±2.5	8.47±1.1	-
18:3(5,9,12)	0.94±0.5	0.30±0.5	0.99±1.7	-	19.7±1.9	38.7±4.0	-
18:3(9,12,15)	33.3±3.3	19.6±5.8	4.36±1.7	7.30±1.4	7.89±1.8	3.90±3.0	2.05±3.6
18:4(5,9,12,15)	-	0.17±0.3	1.31±1.2	0.69±1.2	5.49±0.6	2.18±0.4	3.00±<0.0
C16/C18	1.05	1.12	5.00	1.73	0.70	0.10	1.26
Lipid/TLs (%)	37.8	21.2	6.6	5.7	22.3	5.2	1.2

(%)

Chapter 2

Analysis of TAG synthetic pathway *in C. reinhardtii*

2-1 Introduction

TAG was synthesized mainly in Kennedy pathway (Kennedy 1969)。This reaction goes acyl-translation to *sn*-1, 2, 3 positions in glycerol backbone. Diacylglycerol acyltransferase (DGAT) which is considered as limiting enzyme in this synthetic pathway, carries final step as acylation to *sn*-3 position of DAG. Two types of DGATs were found in many organisms called DGAT type 1 (DGAT1), and DGAT type 2 (DAGT2 or DGTT) (Turchetto-Zolet et al., 2011). In mammal and plant study about DGATs, designates characteristics of these two proteins. DGAT1 which includes 6 or more transmembrane domains, is membrane proteins (Cases et al., 1998). Using land plant, it was reported that DGAT1 expressed in various organs and cells widely, in In *Arabidopsis thaliana*, DGAT1 genes sustain TAG content in seeds (Caboche et al., 1999, Zou et al., 1999). DGAT2 is also membrane protein, in which 2 or 3 transmembrane domains are contained. Overexpression of DGAT2 from *Umbelopsis ramannian* increased TAG amount in *Glycine max*, and *Zea mays* (Lardizaval et al., 2008, Oakes et al., 2011). Functional differences between DGAT1 and DGAT2 is still not clear but studies on DGAT2 indicated that this enzyme may function in lipid developing organs such like seeds (Shockey et al., 2006,, Li et al., 2013). However, these enzymes concern OD formation, since both of these gene mutants in mice did not have any LDs (Harris et al., 2011). Other TAG synthetic proteins are also known. Cytosolic DGAT called DGAT3 was found in *Arachis hypogea*, and *Arabidopsis thaliana* (Saha et al., 2006, Vaistij et al., 2012). Acyl-CoA independent TAG synthesis, is allowed by phospholipid; diacylglycerol acyltransferase (PDAT). PDAT which translates acyl-fatty acid from *sn*-2 position of phospholipid such as PE and PC to *sn*-3 position in DAG, were reported in *Saccharomyces cerevisiae*, *Arabidopsis thaliana* (Dahlqvist et al.,2000, Ståhl et al., 2004), and *Chlamydomonas* (Yoon et al., 2012). Moreover, interestingly, lipase which acts on thylakoid lipid MGDG, translating FA was found in *Chlamydomonas* (Li et al., 2012).

Algae enhance TAG accumulation under various stress conditions. In the chapter 1, the author analyzed TAG content under -S, -N, -P conditions, and indicated the TAG accumulating mechanism which is controlled by stress stimulation differentially. Biogenic metabolism consists of synthesis and degradation and/or elimination. This balance decides mass changing in the cell. Here the author focused on TAG synthetic reaction in Kennedy pathway. Different from mammal and land plants, the algal research on TAG synthetic pathway has been started since 2011. This thesis which is one of its intense fields in algal research, demonstrated synthetic gene analysis, and investigated TAG accumulating mechanisms.

2-2 Material and Methods

DGAT gene analysis and characterization

DGAT homologs in *C. reinhardtii* were picked up using BLAST in The National Center for Biotechnology Information (NCBI http://www.ncbi.nlm.nih.gov/), and Genome Net (http://www.genome.jp/ja/) by from yeast (NP_014888.1, NCBI) and *Arabidopsis* (AT3G51520, KEGG). Multiple alignment and phylogenetic tree were also drawn with CLUSTALW (http://www.genome.jp/tools/clustalw/), BoxShade (http://www.ch.embnet.org/software/BOX_form.html), and MOLECULAR EVOLUTIONARY GENETICS ANALYSIS (MEGA , Tamura et al., 2011) prepared from http://www.megasoftware.net/.

Semi-quantitative determination of transcript levels by reverse transcriptase (RT)-PCR

Total RNA was extracted and purified by phenol–chloroform extraction, as described by Los *et al.* (1997), and then used for cDNA synthesis by reverse-transcription with random primers (Tabei *et al.*, 2007). The cDNA synthesized was used as a template for semi-quantitative RT-PCR (Tabei *et al.*, 2007). The forward (F) and reverse (R) primers used are summarized in supporting information Table S1. The primer sets for *DGAT1* and *DGTT1-4*, *GPDH1-3*, and 18 rRNA were the same as those reported by Msanne et al. (2012), Herrera-Valencia et al. (2012), and Teramoto et al. (2002), respectively (see below). The primer sets for *GPAT1*, *GPAT2*, and *LPAAT* were designed on the basis of their corresponding cDNA sequences (NCBI reference sequences), XM_001694925.1, XM_001696416.1, and XM_001694664.1, respectively. Amplified DNA fragments were subjected to agarose gel electrophoresis, and a fluorescent image of a gel after staining with ethidium bromide was obtained by photography. The fluorescence intensities of DNA bands were quantified with ImageJ (http://rsbweb.nih.gov/ij/). As regards the respective genes, the values were estimated relative to that of 18S rRNA as an internal control.

Primer set

5’→3’

Gene name	Forward	Reverse
DGTT1	GAAGCAGGTGTTTGGCTTCT	CAGTGCCTCCGTGTAGGTCT
DGTT2	GCGCCGCAACATTTACATGG	CAGCCGTACTCGGTCTTGTG
DGTT3	GTCAGAGCCAAGTGCTGGAC	CCACCTCCTTGTCGAACTC
DGTT4	GCATGTTTGGGCAGTACGGC	GCCTTGTGCTTGTCGTACAG
DGAT1	ACTGGTGGAATGCGGCTAC	TAGCAGCTCGTGGAACACAG
GPAT	GTGTGTTTGCCCATATGCTG	CATCATGGGGTAGCTGAACA
GPAT9	CCTGTGGCCATCAAGTACAA	AGATGTCGCACACCAGAGC
LPAT	CGCCCTTCTACAAAGTCACG	CTCACCCTCAGGGAAGAACA
GPDH1	AGATGCGGGCGTTCTCCAAGG	AGCAGGTCCGTCTCCAGGTCC
GPDH2	ATGCTATGCGACCTATCTGC	ACTGCTCCTGCTCATTGTG
GPDH3	ATCGGCTCTGGCGCGAGCAGCAC	ACTGCTCCTGCTCATCGGTGAC
18srRNA	ACTGCTCTGCTCCACCTTCC	TATTCAGAGCGTAGGCCTGC

2-3 Results

DGATs in C. reinhardtii and its characteristics about amino acid sequences.

One DGAT1 and 5 DGAT2s (now called DGTTs in algae) were found in *C. reinhardtii*. DGTT1-4 protein lengths were 320-350 amino acids residue which is common in other DGAT2 proteins (table 2-1). Phylogenetic tree indicated that *C. reinhardtii* possess 3 grouped DGTTs. At first, DGTT1 which had 35% homology with DGA1 protein (Sorger et al., 2002) was belonged in the group of animals. DGTT4 which is the highest homology with atDGAT2 was in land plants group. Thirdly, DGTT2, 3, 5 made an original group as *C. reinhardtii* (Fig. 2-1). These DGTTs were predicted membrane protein as well as other DGAT2s (table 2-2, Fig. 2-2). However, there were differences in the number of transmembrane domains by tools. Regions of 41-63 residues were common, but TMHMM server didn't recognized about 97-119、253-275 residues in DGTT1. Also seen in DGTT5, that is 124-146 and 150-171 regions were not included by TMHMM.

C. reinhardtii DGAT1 (crDGAT1) reserved well known DGAT1 motif HKWXXRHXYXP and active histidine residue (Fig. 2-3) (Mcfie et al., 2010). Former HKWXXRHXYXP binds DAG/phorbol (Oelkers et al., 1998). Multiple alignments of DGTTs supported as active DGAT2 protein possibility (Fig. 2-4). It was widely known that DGAT2 contain three motifs as YFP, HPHG, RXGFX(K/R)XAXXXGXXX(L/V) VPXXXFG(E/Q) (Liu et al., 2011). First YFP was reserved in DGTT1, 4, third proline (P) changes to lysine (K), histidine (H), asparagine (N) in DGTT2, 3, 5 respectively (Fig. 2-4). PH is important for DGAT2 activity in second HPHG residues, and almost of all organisms which have TAG contain this region (Liu et al., 2011). Animal second motif known as HPHG, but there are often found EPHS in plants. All DGTTs have PH residues but first residues were changed to phenylalanine, serine, cysteine. The half of third motif as RXGFX(K/R)XAXXXG highly accorded but latter XXX(L/V)VPXXXFG(E/Q), L/V was replaced to isoleucine (I) in DGTT2, 3, 5, besides DGTT3, 222 proline was changed to cysteine. Finally, DGTT1 and DGTT5 were not phenylalanine in 192 tyrosine and 274 methionine respectively.

Gene expression analysis in Kennedy pathway

DGATs and other acyl-transition enzymes in Kennedy pathway described as glycerol-3-phosphate: acyltransferase (GPAT), and lyso-phosphatidic acid: acyltransferase (LPAAT), moreover phosphate dehydrogenase (GPDH) which gives glycerol-3-phosphate (G3P) from dihydroxyacetone phosphate (DHAP) in glycolysis. These 5 classes and 10 genes were investigated the expressions with RT-PCR. Results were shown in pictures and numerically (Fig. 2-5). At first, about *DGATs*, *DGAT1* expressed every condition. Picture looked likely to increase in -N and -P but, there was no numerical significant difference. *DGTTs* was

increased clearly after 8h in -S and -N. Interestingly every genes detected in -S were more clearly than that of in -N. *DGTT5* has not been detected. This result was according to other reports in -N (Msanne et al., 2012, Boyle et al., 2012). DAG synthesis in Kennedy pathway carried by *GPAT1* and *LPAAT* was up-regulated under -S and -P but not -N. These genes code chloroplast localized protein with targeting peptide. GPAT2 which does not have targeting peptide, so that considered as cytosolic GPAT. This *GPAT2* gene did not show any expression pattern changes. Finally, *GPDH* showed similar pattern to *DGTTs*, increasing in -S and -N. *GPDH1* didn't detected in any conditions.

Table 2-1. DGTT proteins homology with DGAT2

	atDGAT2	DGTT4	DGA1p	length
atDGAT2	-	37		314
DGTT1	28	31	35	320
DGTT2	30	30	25	324
DGTT3	34	28	25	346
DGTT4	38	-	30	327
DGA1p	30	30	-	418

Protein sequence homology (%)

Amino acid (aa) length

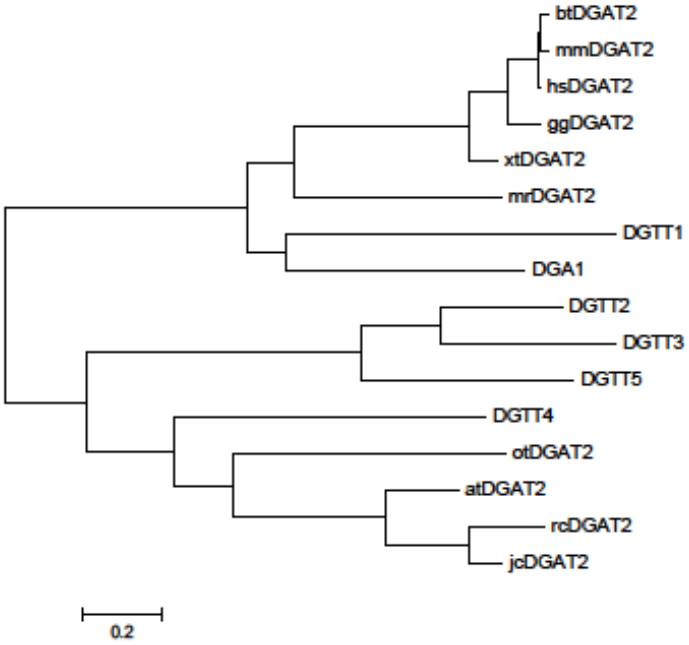


Fig. 2-1. Phylogenetic tree of DGAT type 2s (DGTTs) was described by MEGA software (Tamura et al., 2011) with maximum likelihood estimation. Amino acid sequences were prepared from NCBI (http://www.ncbi.nlm.nih.gov/) btDGAT2: *Bos Taurus* (NP_991362.2). mmDGAT2: *Mus musculus* (NP_080660). hsDGAT2: *Homo sapiens* (AAQ88896). ggDGAT2: *Gallus gallus* (XP_419374.3). xtDGAT2: *Xenopus tropicalis* (NP_989372.1). mrDGAT2: *Umbelopsis ramanniana* (Q96UY2.1). DGA1: *Saccharomyces cerevisiae* S288c (NP_014888.1). otDGAT2: *Ostreococcus tauri* (XP_003083539.1). atDGAT2: *Arabidopsis thaliana* (NP_566952.1). rcDGAT2: Ricinus communis (XP_002528531.1). jcDGAT2: Jatropha curcas (AEZ56254.1) DGTT: *Chlamydomonas reinhardtii* (DGTT1:CHLREDRAFT_180240, DGTT2:CHLREDRAFT_184281, DGTT3: CHLREDRAFT_188937, DGTT4: CHLREDRAFT_190539).

Table 2-2. Prediction of Membrane Proteins in DGTTs

	type	helices	region
DGTT1	MEMBRANE PROTEIN	3	41-63,97-119,253-275
DGTT2	MEMBRANE PROTEIN	2	26-48,55-77
DGTT3	MEMBRANE PROTEIN	3	49-71,78-100,149-171
DGTT4	MEMBRANE PROTEIN	2	11-33,43-65
DGTT5	MEMBRANE PROTEIN	4	33-55,62-84,124-146,150-171

SOSUI server (http://bp.nuap.nagoya-u.ac.jp/sosui/)

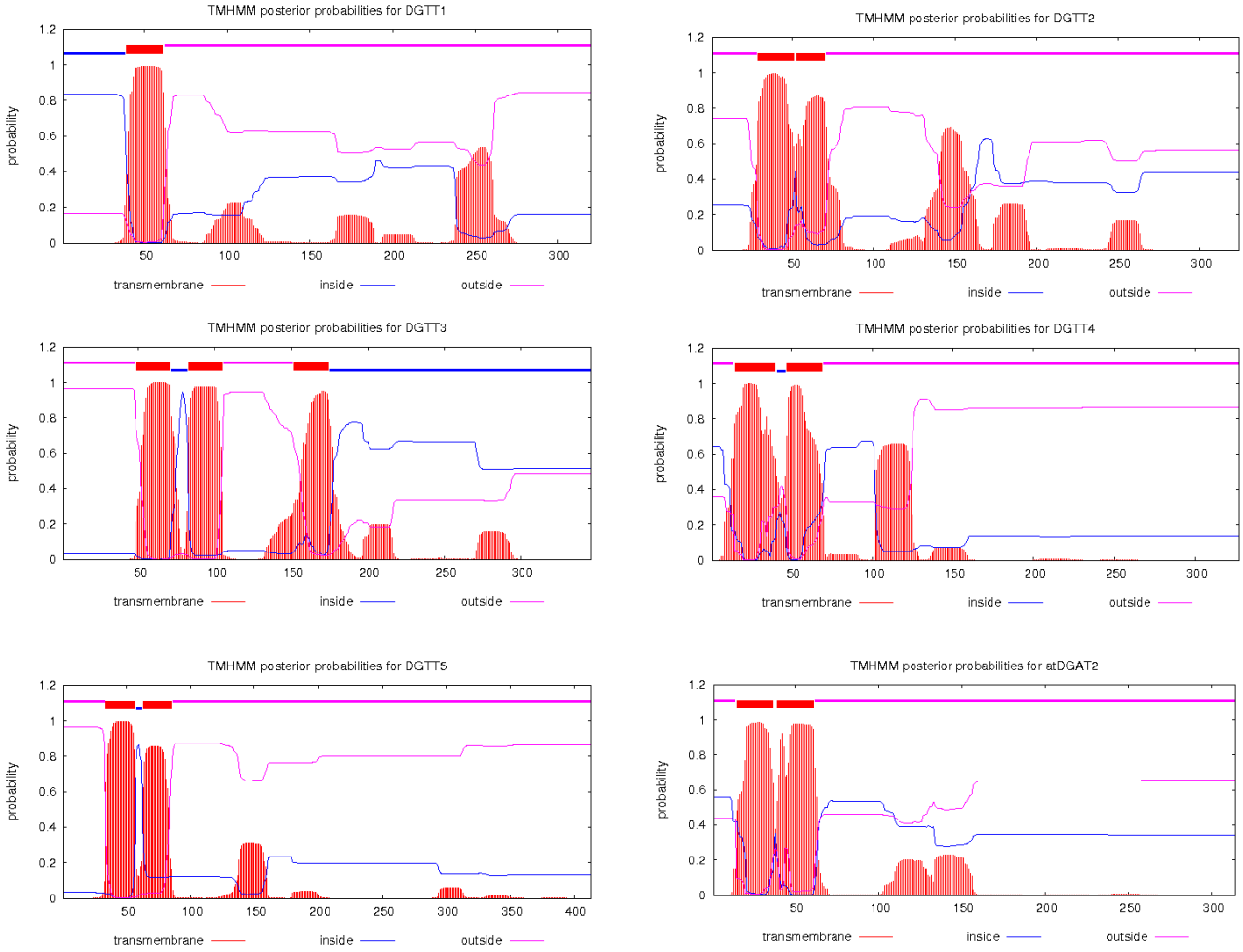


Fig. 2-2. Prediction of transmembrane helices in DGAT type2s. TMHMM Server was used for analysis. Red bold lines indicate transmembrane site, blue lines shows inside, red lines means outside residues.

DGAT1

Fig. 2-3. Multiple alignment of DGAT1 and its conserved motif. DGAT1 homologs were studied in various organisms. HKWXXRHXYXP sequence is diacylglycerol/phorbo ester binding site, which conserved in crDGAT1. *Histidine residue is reported as active site was also found in this homolog. crDGAT1: *C. reinhardtii*, mmDGAT1: *Mus musculus*. hsDGAT1: *Homo sapiens*. atDGAT1: *Arabidopsis thaliana*. tmDGAT1: *Tetraena mongolica*. ptDGAT1: *Phaeodactylum tricornutum*.

DGAT2

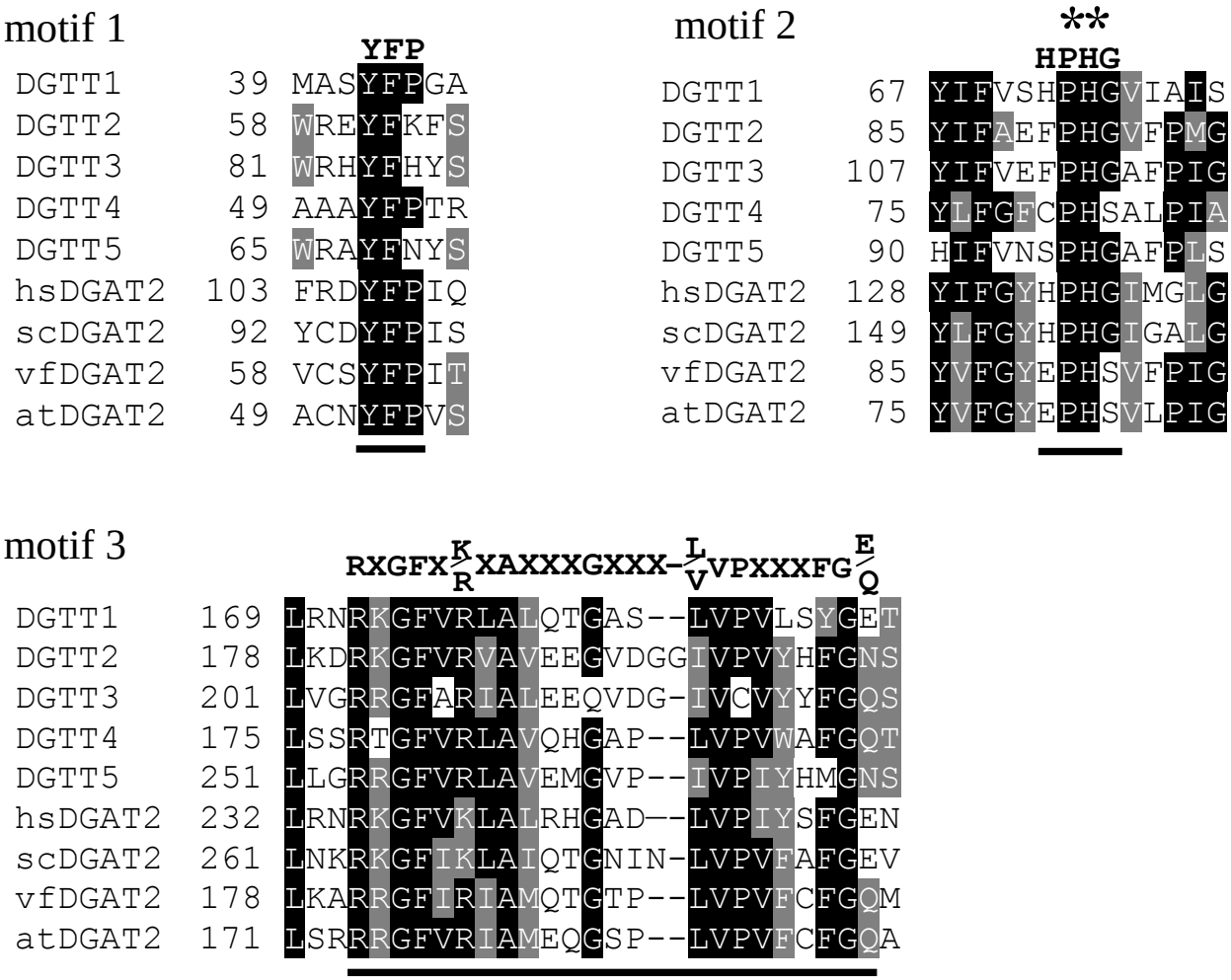


Fig. 2-4. Multiple alignment of DGAT2 and its conserved motif. Three motifs were previously characterized in DGAT2. YFP, HPHG, and RXGFX(K/R)XAXXXGXXX(L/V)VPXXXFG(E/Q). Second motif especially *PH is considered as important residues for normal function of DGAT2. hsDGAT2: *Homo sapiens*. scDGAT2: *Saccharomyces cerevisiae*. vfDGAT1: *Vernicia fordii*. atDGAT2: *Arabidopsis thaliana*. ptDGAT1: *Phaeodactylum tricornutum*.

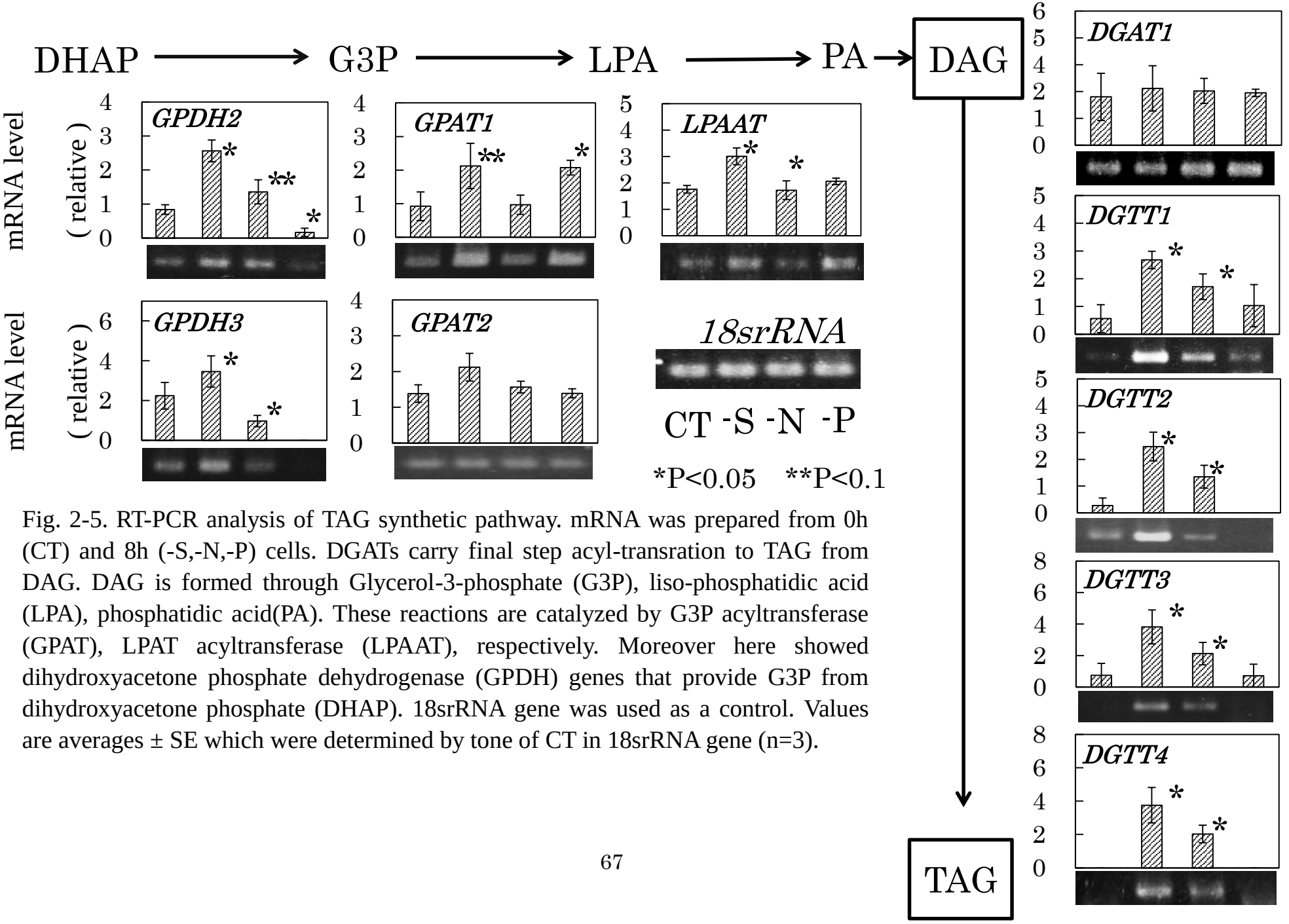


Fig. 2-5. RT-PCR analysis of TAG synthetic pathway. mRNA was prepared from 0h (CT) and 8h (-S,-N,-P) cells. DGATs carry final step acyl-transration to TAG from DAG. DAG is formed through Glycerol-3-phosphate (G3P), liso-phosphatidic acid (LPA), phosphatidic acid(PA). These reactions are catalyzed by G3P acyltransferase (GPAT), LPAT acyltransferase (LPAAT), respectively. Moreover here showed dihydroxyacetone phosphate dehydrogenase (GPDH) genes that provide G3P from dihydroxyacetone phosphate (DHAP). 18srRNA gene was used as a control. Values are averages ± SE which were determined by tone of CT in 18srRNA gene (n=3).

2-4 Discussion

C. reinhardtii owned one DGAT1 and five DGTTs which were classfied into three groupes as animal, land plant, and original *C. reinhardtii* types (Fig. 2-1). This result corresponded with previous report (Russa et al., 2012). They were membrane proteins and possesed common motifs in both of DGAT1 and DGTTs separatly. YFP residues that is first motif in DGTTs, seemed to locate in transmembrane domain (Table 2-1, Fig. 2-2). This is also known in other DGAT2. DGAT2 activity depend on histidine at second motif 'HPHG'. Site directed mutation of second histidine to alanine in yeast abolished activiy (Liu et al., 2011). In spite of the importance, this motif shows variety. It was repoted that this motif reserved as HPHG in fungi and animals, as EPHS in land plants (Liu et al., 2012). Althofh first His residue changed to unique amino acids such as phenylalanine (F), cysteine (C) and serine (S) in 'HPHG', DGTTs had important 'PH' residues in common. These charactheristics in amino acid residues indicated that DGATs in *C. reinhardtii* function TAG synthesis. Actually, recent studies have demonstrated TAG synthetic activity about DGTTs. Overexpressions of *DGTT1-3* in *C. reinhardtii* increased TAG content (Russa et al., 2012), and Hung et al reported that *DGTT1-3* transgene to TAG defective yeast recovered TAG product capacity, especially in *DGTT3* inducted mutant (Hung et al., 2013).

RT-PCR analysis suggested that *DGTTs* contribute to TAG accumulation by gene up-regulation under stressed condition. However, each *DGTT* expressed higher in -S than in -N. This is a disagreement with that produced TAG amount was higher -N than -S. This result indicated that the levels of gene expressions would not determine TAG content simply. On the other hand, *DGAT1* gene did not change in any conditions. There are distinct results about *DGAT1* expression. Msanne et al insisted that *DGAT1* gene wouldn't response to N-starvation (Msanne et al., 2012), but Boyle et al showed *DGAT1* gene up-regulating under -N condition by quantitative RT-PCR (Boyle et al.,2012). In Boyle issue, there may be affects by growth conditons such as carbon source or by cell specific differences. As my conclusion in this thesis, *DGAT1* gene is not response to stressed conditions at least, because gene expression hardly change and kept certain levels. There may be time-dependent alterlations of the levels of *DGAT1* transcription.

DAG synthesis was promoted by gene-expressions which code acyl-translatite enzyme under -S and -P conditions. Therefore, S-deficient TAG accumulation which accompanys DAG and TAG gene up-regulations, was distinguished from N-deficient TAG accumulation. Moreover, DAG synthesis was promoted in chloroplastidic reaction. It was to explain by demand for polar lipids synthesis. Thylakoid membrane lipid synthesis also undergoing and increase PLs under S and P starved conditions, (see supplemental Fig. 1-c). Whereas, FA synthesis under -N condition

was reported by Ramanan et al 2013. Genes of acetyl-CoA carboxylase and malonyl-CoA ACP transacylase which contribute FA synthesis were down-regulated.

Thus, it was considered that DGATs in *C. reinhardtii* carries TAG synthesis and *DGTT*s up-regulated in -S and -N conditions and there were different lipid change mechanisms in gene expressions of Kennedy pathway. *DGTT* gene expressions were important for TAG accumulation, but there is other factor to determine TAG content.

Chapter 3

TAG accumulating mechanisms and participation of *SAC* genes

3-1 INTRODUCTION

TAG accumulation under -S and -N conditions were cooperated with gene up-regulations, but its regulation wouldn't decide TAG amounts. There are other mechanisms which determine TAG content in the cells. Furthermore, it is not still clear that physiological meaning of TAG accumulation in severe environment such as -S, -N and RAD conditions. Results from chapter 1, the author noted that S and N compose proteins. Abnormal protein metabolisms may concern to TAG accumulation. TAG plays a role of energy storage mainly. G3P is the first substance in Kennedy pathway, and is originated from reduction of DHAP in glycolysis and TCA cycle. Surplus low molecular carbon compounds which are in the above chemical and reductive energy producing reactions, preserved as lipids or carbohydrate. Thus, the author supposed that abnormal protein metabolism makes surplus energy in the cells, and carbon compounds proceed to TAG synthesis. In this chapter, to understand TAG accumulating mechanism, it was studied that the effect of inhibitors of protein-, FA-, and photo- syntheses and of mutants on TAG accumulation.

Meanwhile, there is well known factor which controls sulfur starvation response, described as SAC (sulfur acclimation). Two *SAC* gene mutants have been isolated and studied. SAC1 which is homologous to the $Na+/SO_4^{2-}$ transporter locates in membranes with 10 transmembrane domains, but function as a sensor for sulfur. *Δsac1* mutant shows high sensitivity to -S condition and sulfur deprivation responsive genes suppression (Wykoff et al., 1998). On the other hand, SNRK 2.2 [SNF1-related protein kinase 2.2, previously known as SAC3 (Moseley et al., 2009)] protein belongs to the serine/threonine kinase group, and seems to either positively or negatively regulate physiological responses related to the ambient S-status (Zhang et al., 2004, Moseley et al., 2009). *Δsnrk2.2* has general arylsulfatase (ARS) activity that activated in S-shortage (Davies et al., 1999, Gonzalez-Ballester et al., 2008). The mechanism by which the accumulation of TAG is stimulated under S-starved conditions will be discussed, in the view of the actions of the *SAC1* and *SNRK2.2* genes.

3-2 Materials and Methods

Strains and growth conditions

C. reinhardtii was prepared for lipid, starch, and protein analysis. Cells were cultivated with TAP medium described in chapter 1. CC125 cell as the wild type, and three disruptants *SAC1*, *SNRK2.2*, and *ARG9* genes, and the respective complemented strains were prepared (Remacle et al., 2009). These mutants and complemented strains were purchased from *Chlamydomonas* Resource Center (http://chlamycollection.org/). A disruptant of the *ARG9* gene (*Δarg9*), which is deficient in arginine synthesis owing to the impaired gene for N-acetyl ornithine aminotransferase in chloroplasts, was cultured with supplementation of arginine (0.57 mM, Remacle et al., 2009)

Strain	Description	
Δsac1	*C. reinhardtii*	*CC-4353 sac1::ARG7 mt*
Δsac1/SAC1	*C. reinhardtii*	CC-3795 sac1::ARG7 C12-SAC1 complemented mt+
Δsnrk2.2	*C. reinhardtii*	CC-4354 sac3 mt+
Δsnrk2.2/SNRK2.2	*C. reinhardtii*	CC-3798 sac3::ARG7 cos2-2-SAC3 complemented
Δarg9	*C. reinhardtii*	CC-4440 arg-2 mt+
Δarg9/ARG9	*C. reinhardtii*	CC-4441 arg9-2::ARG9 mt+

Inhibitors of protein synthesis, fatty acid synthesis, and photosynthesis

Chloramphenicol (CAP, 310μM), cycloheximide (CHI, 28μM), cerulenin (10 □M), or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, 50 □M), amiprophos-methyl (APM, 10μM) were added immediately after the change of nutrient-replete to –deplete medium, as described by Sugimoto et al. (2010). Methanol was used as a carrier of CAP (final concentration, 1%). CAP and CHI are inhibitors of de novo protein synthesis on chloroplast and mitochondrial 70S ribosomes, and cytoplasmic 80S ribosomes, respectively. Cerulenin is an inhibitor of de novo synthesis of fatty acids, whereas DCMU inhibits photosynthesis. APM prevents tubulin synthesis which has been used to stop cell division or cell cycle.

Protein extraction and analysis

Whole-cell extracts were prepared through disruption of cells in an extraction buffer (50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100) with a Beads Crusher μT-12 (TAITEC, Japan), and thereafter the protein contents were measured with a BCA assay kit (Pierce, USA).

3-3 Results

Effect of inhibitors and light on TAG content

To demonstrate TAG accumulating mechanism, TAG amount depend on cell mass was compared among physiological process effective processes after 24h (Fig. 3-1). TAG contents under -S condition was increased 3-fold as high as in CT. However, TAG accumulation was suppressed by CHI, cerulenin, DCMU, and under the dark. CHI, cerulenin and DCMU inhibit protein-, FA-, and photo- synthesis. In addition to these results, -S-N double stress conditions and APM which inhibits cell-division did not accumulate TAG, but APM-S was shown huge 7.2-fold TAG increasing relative to initial volume.

TAG accumulation and DGATs gene expressions in SAC mutants

C. reinhardtii cc125 as a wild type grew 2.1-fold in -S condition after 120h, but *Δsnrk2.2* and *Δsac1* showed 3.4 and 1.6 -fold respectively. *SAC* gene complemented strains *Δsac1/SAC1* and *Δsnrk2.2/SNRK2.2* recovered growth level to wild type (Fig. 3-2).

LDs were observed in SAC mutants by microscopy (Fig. 3-3). SAC mutants possessed small but much LDs. As well as cc125, a bowl type structure of chloroplast was disintegrated except *Δsac1/SAC1*. *Δsac1/SAC1* cells retained bowl shaped chloroplast, and LDs located along with its form.

TAG contents relative to TLs were not changed by *SAC1* or *SNRK2.2* mutagenesis. However *SAC1*gene complemented strain showed quick and high TAG accumulation as 53.2% at 72h, and shifted after 120h, *Δsnrk2.2* also increased TAG amount and reached 57.9% (Fig. 3-4). After 120h *Δsnrk2.2* cell contained 203μM TAG amounts remarkably (Fig. 3-5), that is more two times larger than other SAC mutants or WT as 88μM (Fig. 1-6).

Gene expressions of *DGTT*s in *Δsac1* and *Δsnrk2.2* accorded to their TAG contents. *DGAT1* gene expressed in WT and *Δsac1*, but in *Δsnrk2.2* declined to undetectable level. Meanwhile, *Δsnrk2.2* showed clearer *DGTT*s expressions than WT but *Δsac1* had low expressions (Fig 3-6).

TAG accumulation in Arginine requirement mutant Δarg9

Eliminating arginine (ARG) to *Δarg9* made LDs. Blocking the ARG assimilation was so severe that nearly a half of cells died, but the dead cell also owned LDs (Fig. 3-7). FAs quantitative analysis by GC was shown in Fig. 3-8. In spite of cell death, TAG content was actually increased at 24h. After that it diminished remarkably. Cell death was evaded by adding ARG, and cells kept TAG volume till 120h. There was tendency to decline TAG content per TLs to add ARG described as 19.1% to 9.3% in 3μl to 10μl.

Quantitative analysis of protein contents in nutrient-deprived condition and ARG mutants

Total protein content per liquid culture at 72h was measured in each condition (Fig. 3-9). Protein content increased to 6.7-fold with TAP medium. However -S and -N conditions suppressed and it were 1.2, 0.9 -fold respectively. Cells under -P medium synthesized proteins than in -S and -N conditions, but the value was 2.5-fold which decrease 65% compared with TAP. As well as *Δarg9* held protein increasing in ARG-deficient conditions.

Effect of CHI an inhibitor of protein synthesis, on carbon storage level in nutrient replete medium

To investigate carbon fixation flow toward carbon compounds, CHI was added to TAP medium. Cell growth and Chl were observed from 5.0μg/ml in visual (Fig. 3-10), and these cells obtained much more LDs and starch than 0.0μg/ml. The size of LDs was smaller than that of in -S or -N, but the number of LDs was increased to 3.69 from 0.93 in 10μg/ml and 0.0μg/ml (Fig. 3-12, table 3-1).

Green algae have two types of starch called stroma starch and pyrenoid starch which are synthesized in chloroplast. Storma starch was observed in each condition. However, pyrenoid starch developed form 1.0μg/ml CHI. In 10μg/ml CHI, cells contained clear pyrenoid starch contrast to no CHI addition.

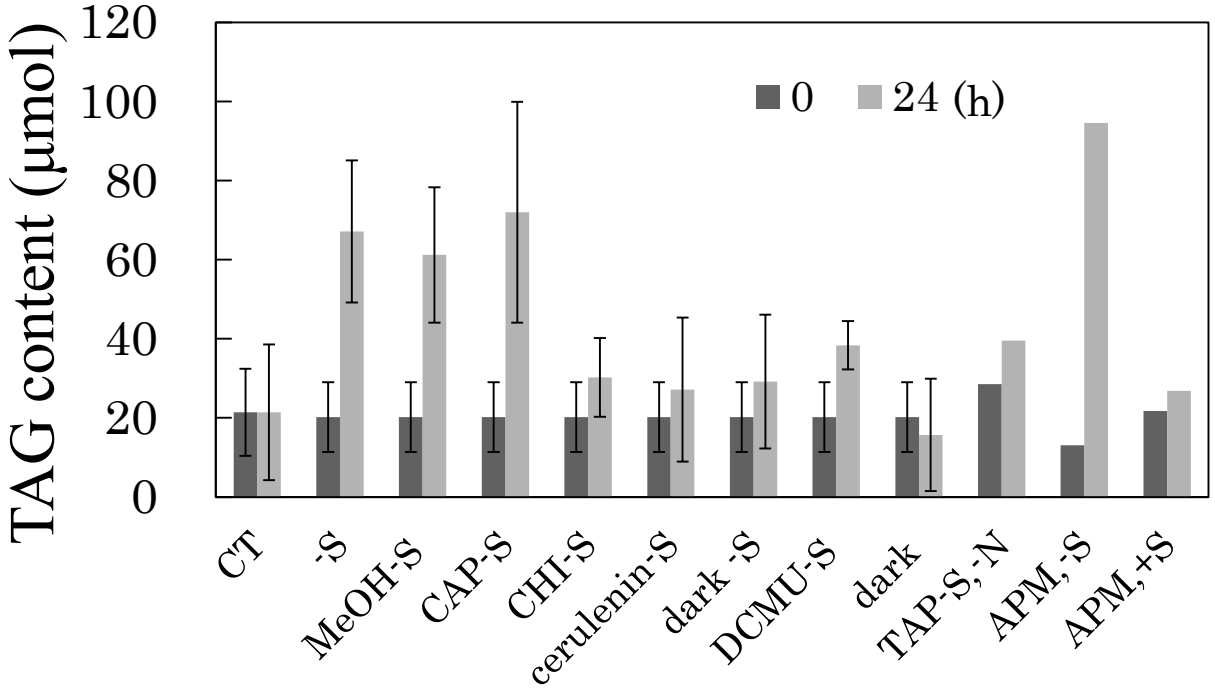


Fig. 3-1. Influence of inhibitor or dark conditions to TAG accumulation after 24h in *C. reinhardtii*. MeOH (methanol) was used for CAP (chloramphenicol) which inhibits chloroplastic proteins synthesis career. CHI (cyclohexymide), cerulenin, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), APM (amiprofos-methyl), inhibit genome nuclear proteins synthesis, de novo FA synthesis, photosynthesis, cell division, respectively. Covered erlenmeyer flask with aluminum foil to make dark conditions. TAG amount was related to cell volume determined as OD_{730} * culture (ml) = 1.0. Values are averages ± SE for three independent experiments from, CT to dark conditions. TAP-S,-N and APM ± S without SE are averages for two experiments.

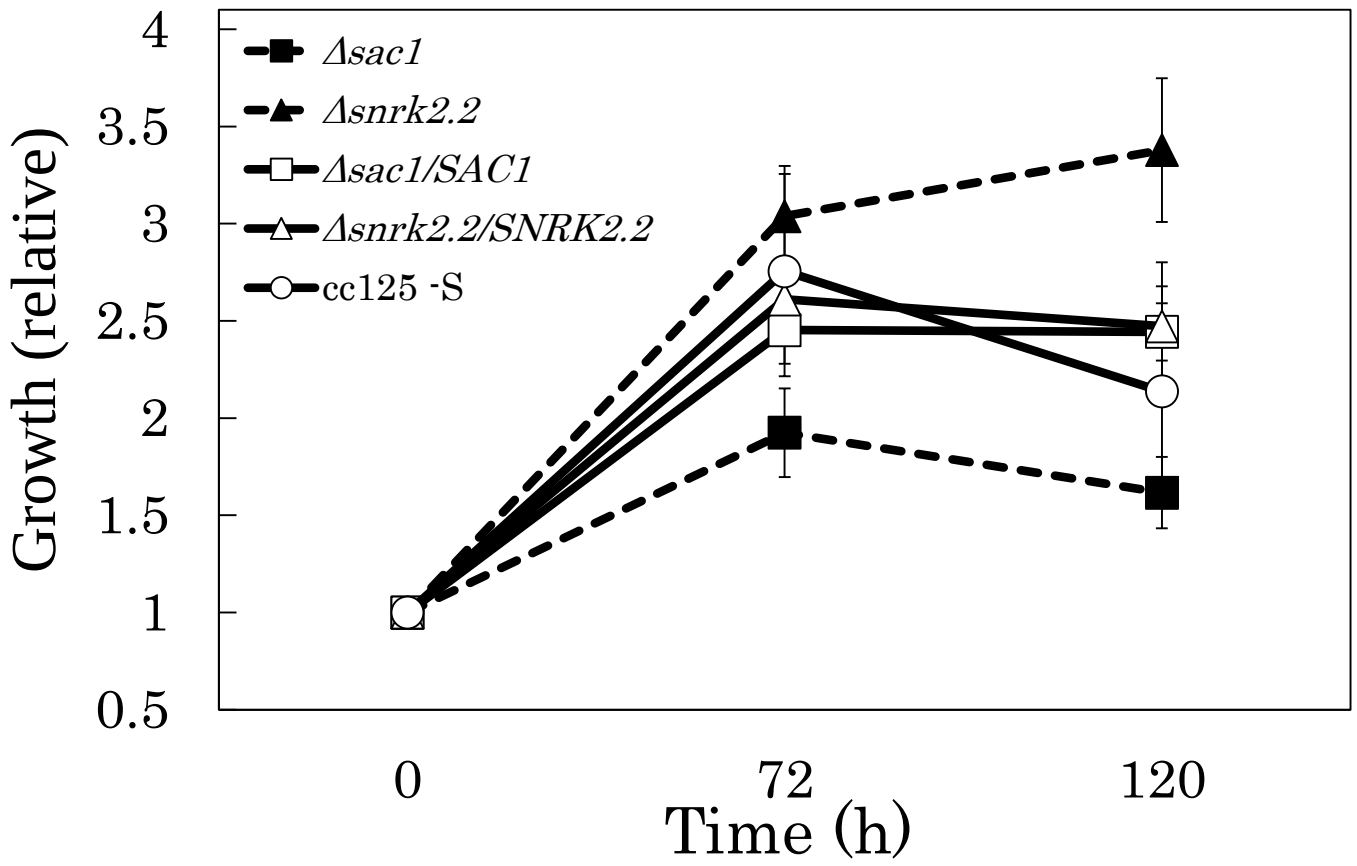


Fig. 3-2. Growth rates of *SAC* mutants which were based on each initial value in TAP-S. *Δsnrk2.2* (closed triangles) showed higher growth than wild type (open circles), and gene complemented strain *Δsnrk2.2/SNRK2.2* (open triangles). *Δsac1* mutant (closed squares) showed lower growth than wild type or *Δsac1/SAC1* (open squares). Values are averages ± SE for three independent experiments

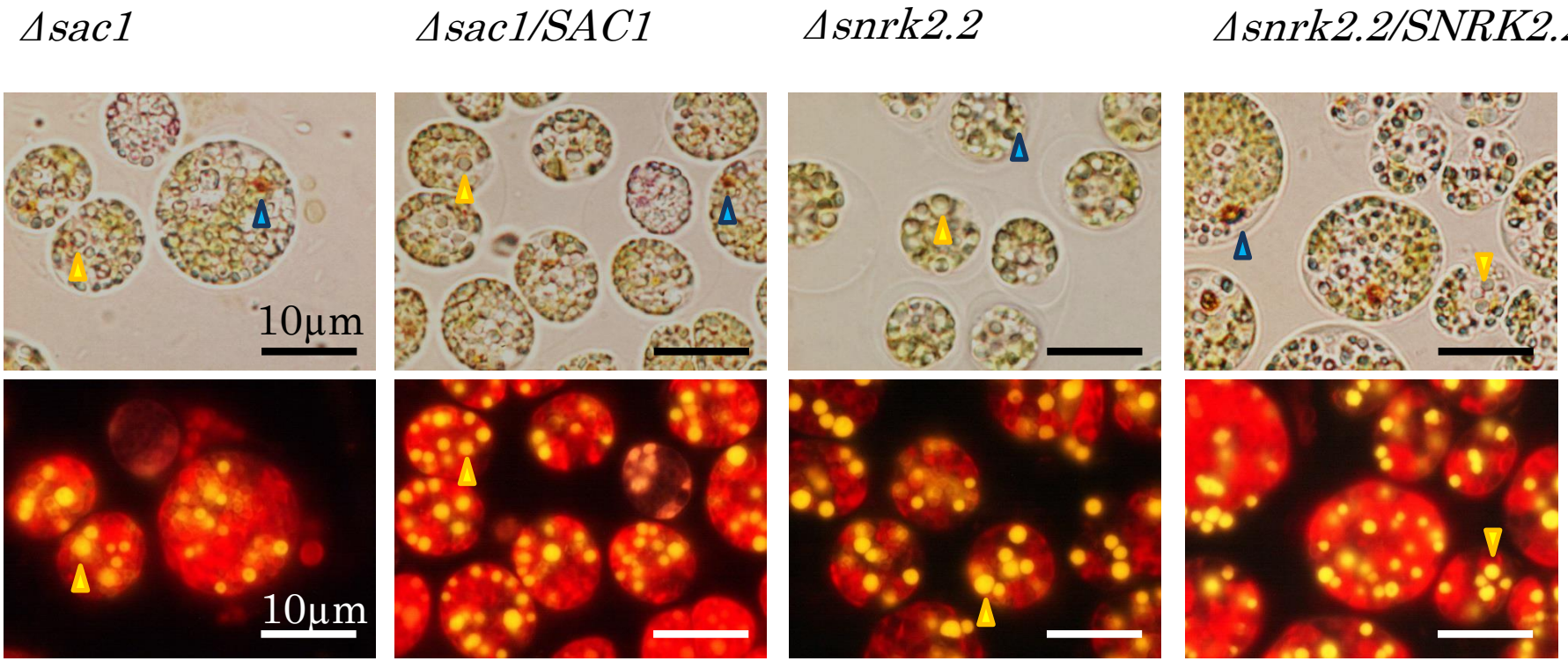


Fig. 3-3. Lipid droplets in *SAC* mutants under sulfur starved conditions 120h. LDs (yellow triangles), eyespot (blue triangles). LDs were stained by Nile Red and observed under excitation lite (520-550nm)

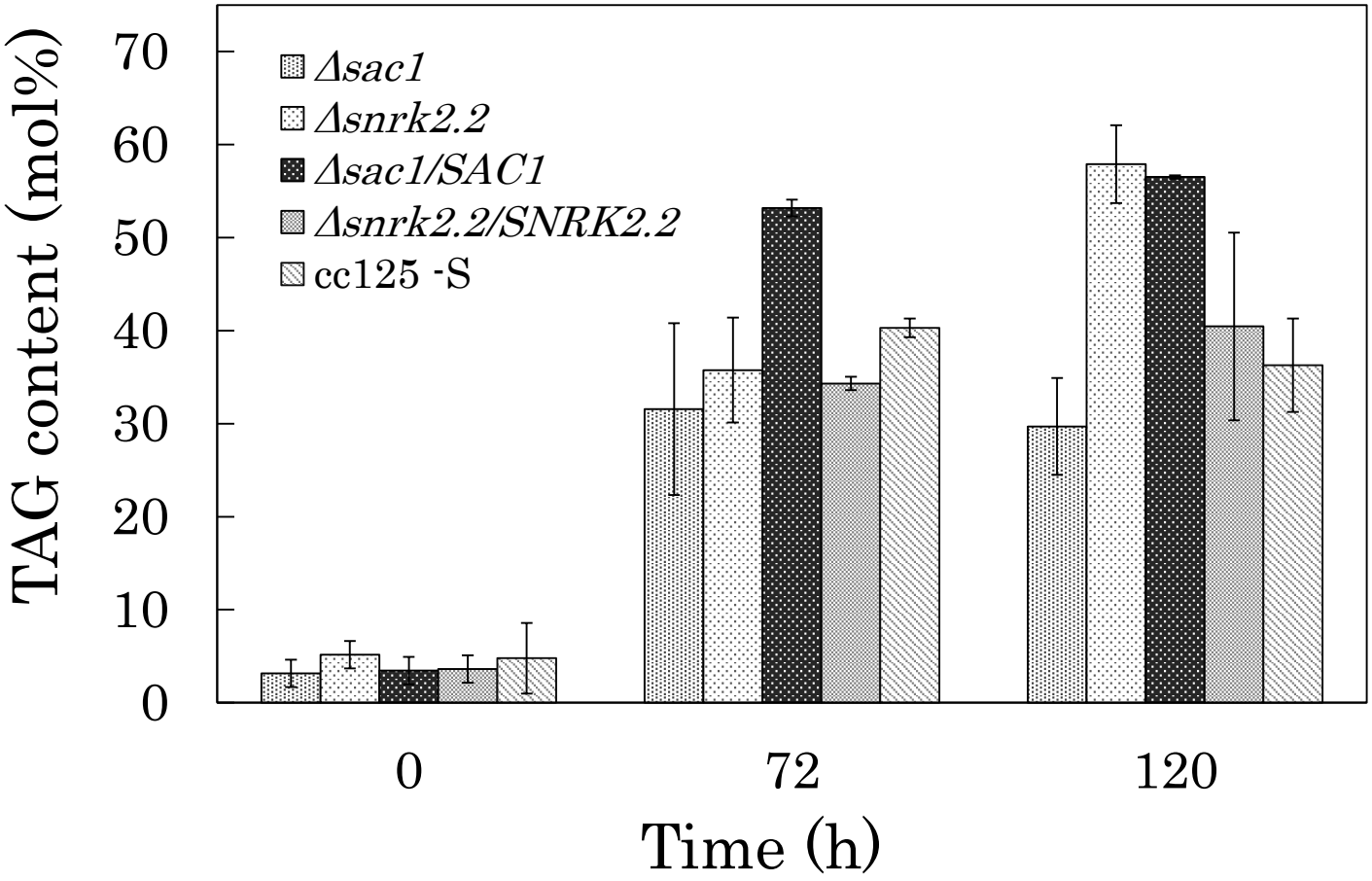


Fig. 3-4. Effects of *SAC* genes on TAG accumulation relative to total lipids (TLs). Lipid content was based of FAs from TAG, or TLs. Values are averages ± SE for three independent experiments

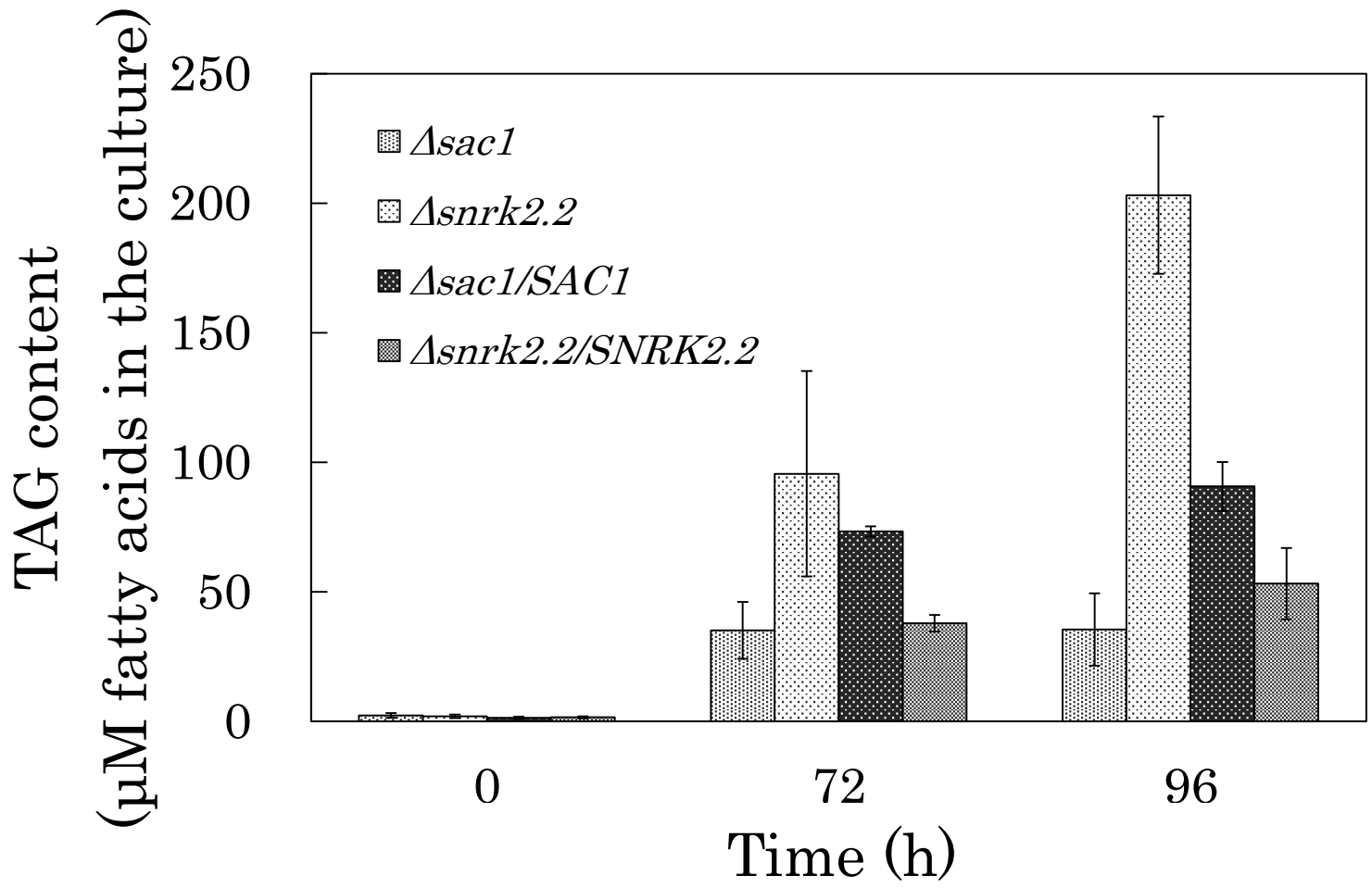


Fig. 3-5. Effects of *SAC* genes on TAG content relative to culture. Lipid content was based of FAs fromTAG. Values are averages ± SE for three independent experiments

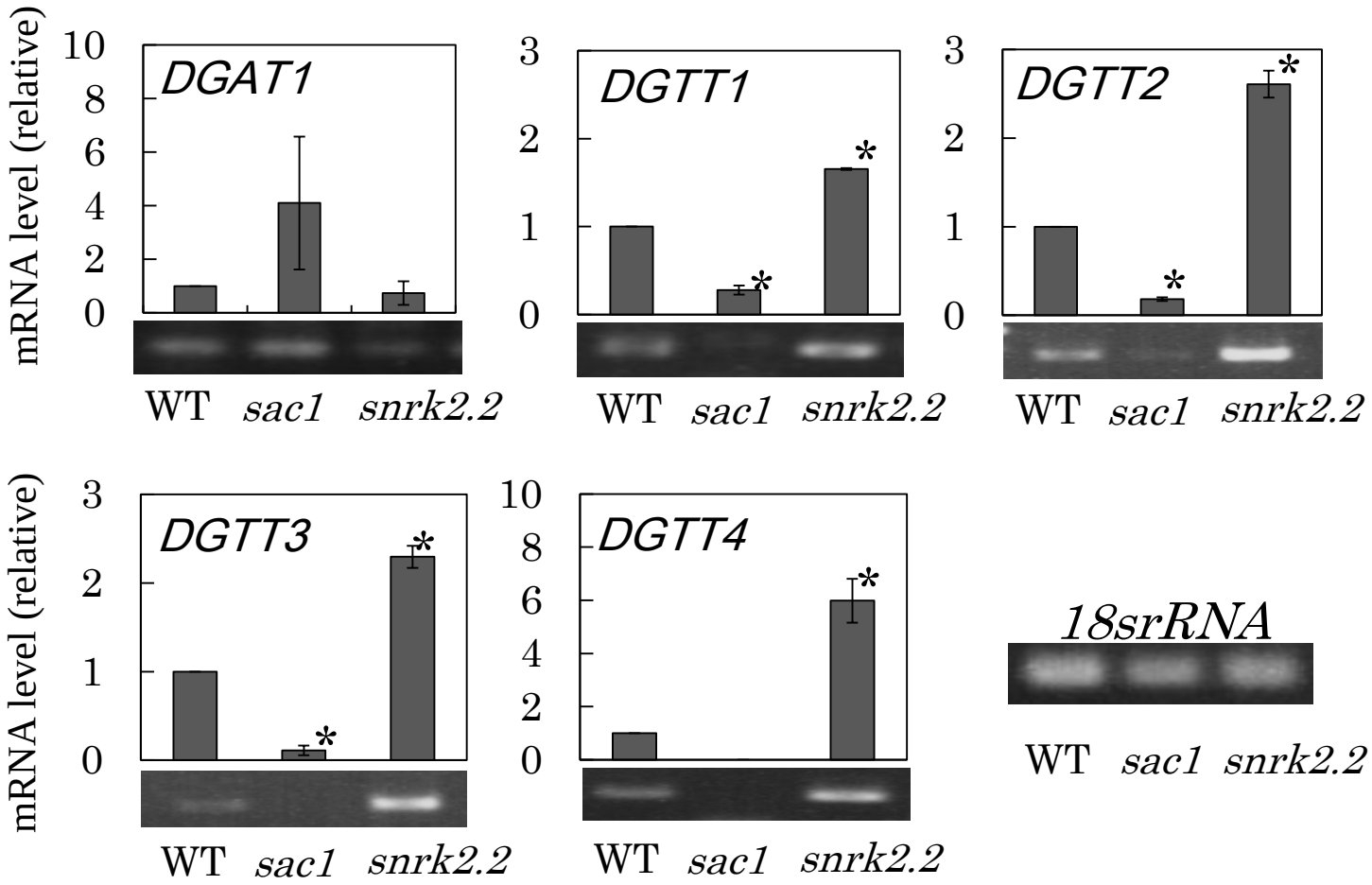


Fig. 3-6. Gene expressions of DGATs in *SAC* mutants by semi-quantitative RT-PCR. mRNA was extracted from cell s 8h after sulfur-deprived conditions shift. Values are based on WT, and shows averages± SE by three experiments. * $p<0.05$

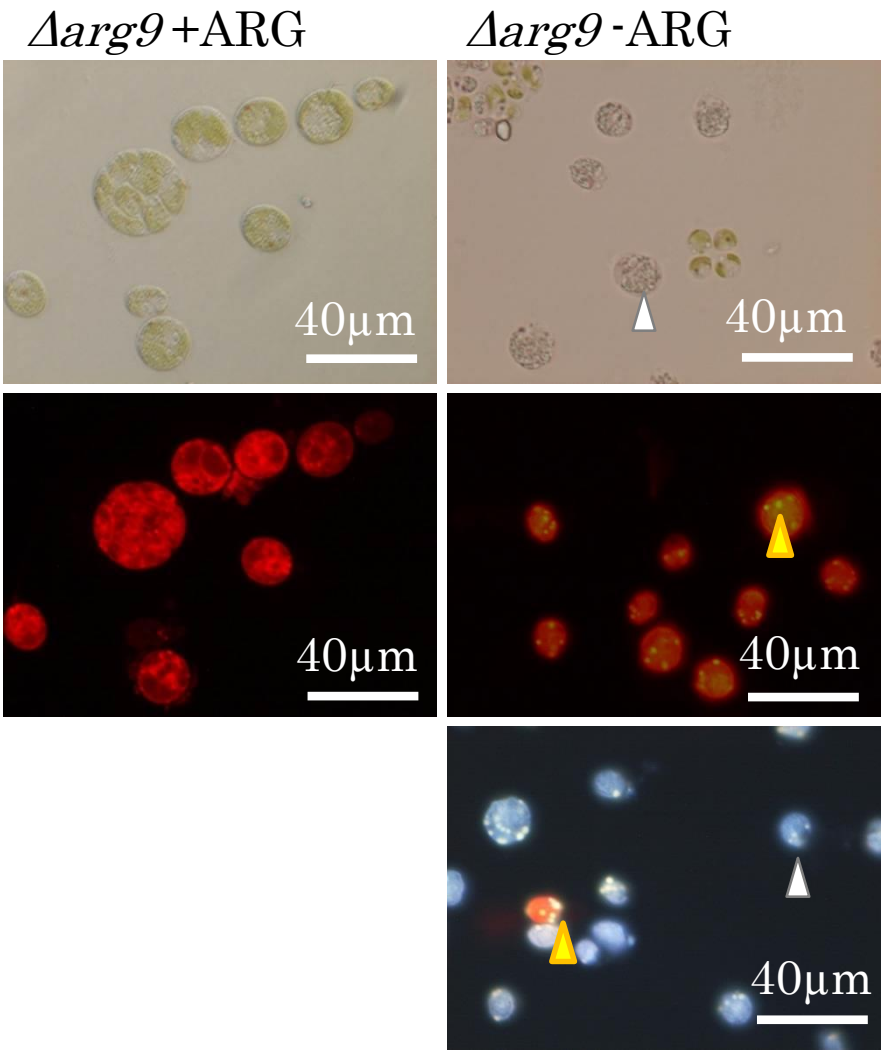


Fig. 3-7. *ARG* mutant observation in TAP ± arginine (ARG) after 96h by microscopy. LDs (yellow triangles), some sells were dead (white triangles) LDs were stained by Nile Red and observed under excitation lite (520-550nm)

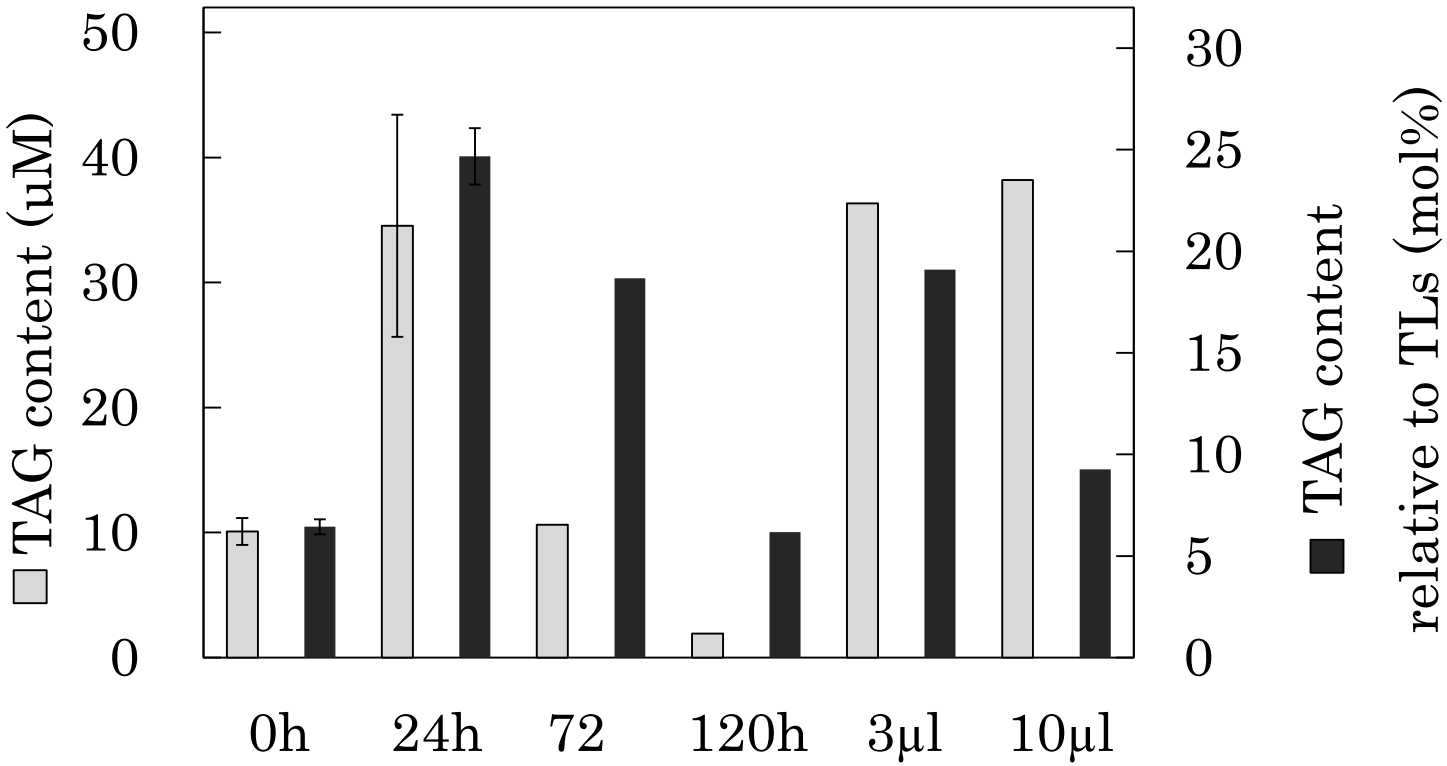


Fig. 3-8. Lipid accumulation of *ARG9* mutants in TAP-ARG until 120h. TAG content relative to cell volume (gray bars), and TLs (black bars). 0-24h show averages ± SE by tree experiments. Other values are only result by one experiment. Adding arginine was able to get a little increase after 120h (3µl : 57.5µM, 10µl :191µM arginine)

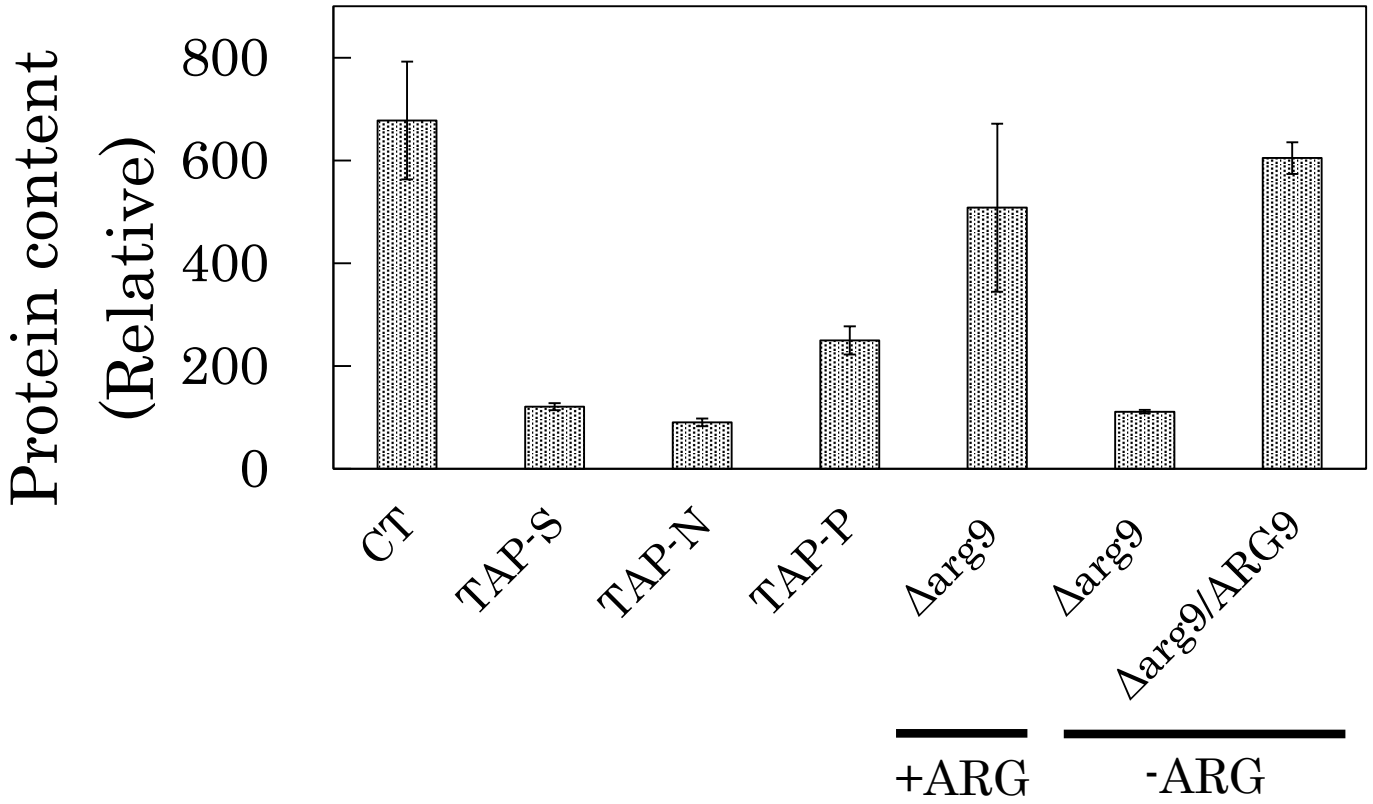


Fig. 3-9. Protein content relative to culture (72 h). Total protein was extracted from cell s by beads crusher with tritonX-100, and measured by BCA assay with BSA standard. Set the initial volumes of protein to 100. Values are averages ± SE for three independent experiments.

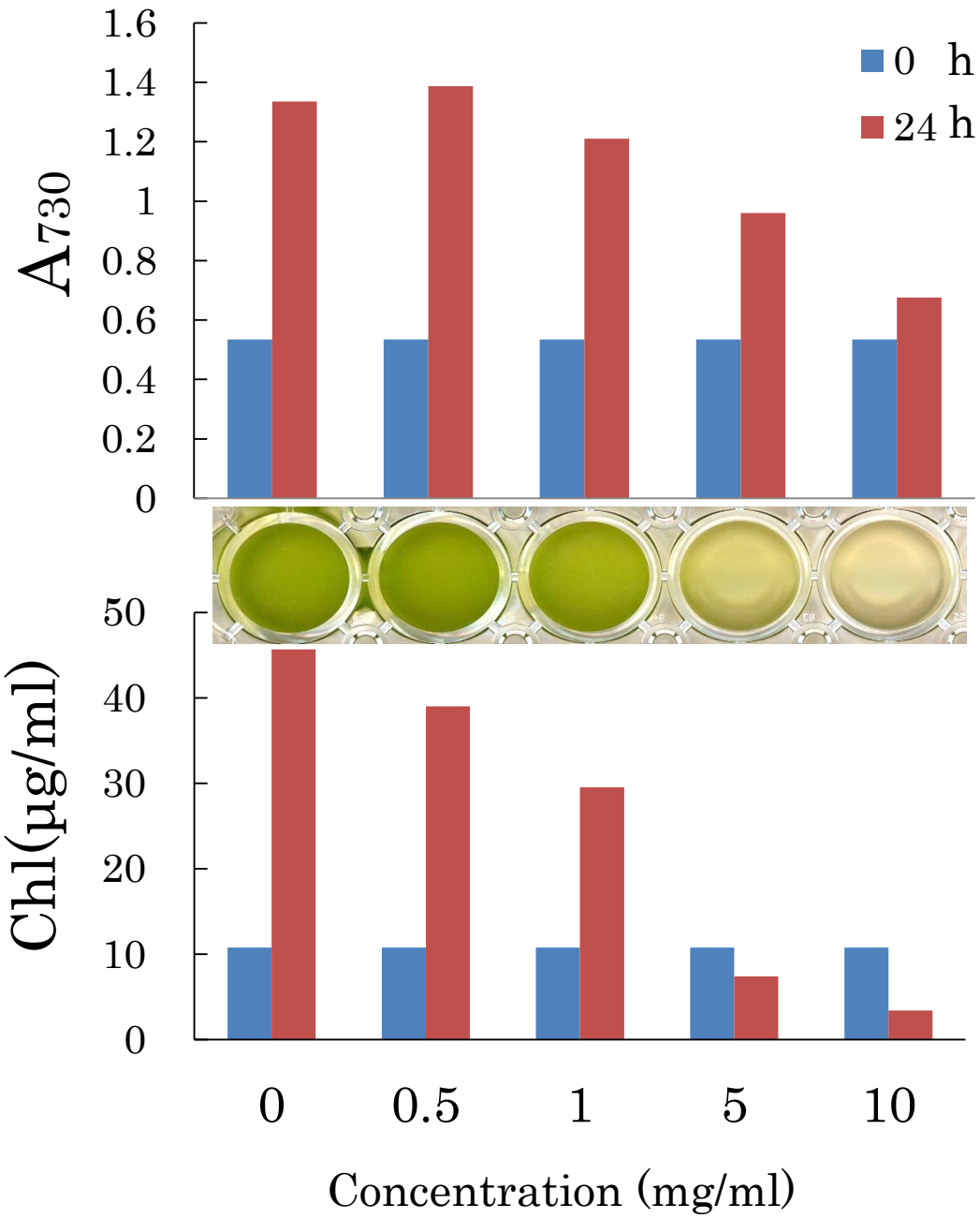


Fig. 3-10. Growth gradation by concentration of cyclohexymide in TAP. Cyclohexymide was added after culture refreshing with each concentration, after 24h, absorbance 730nm and Chl were measured.

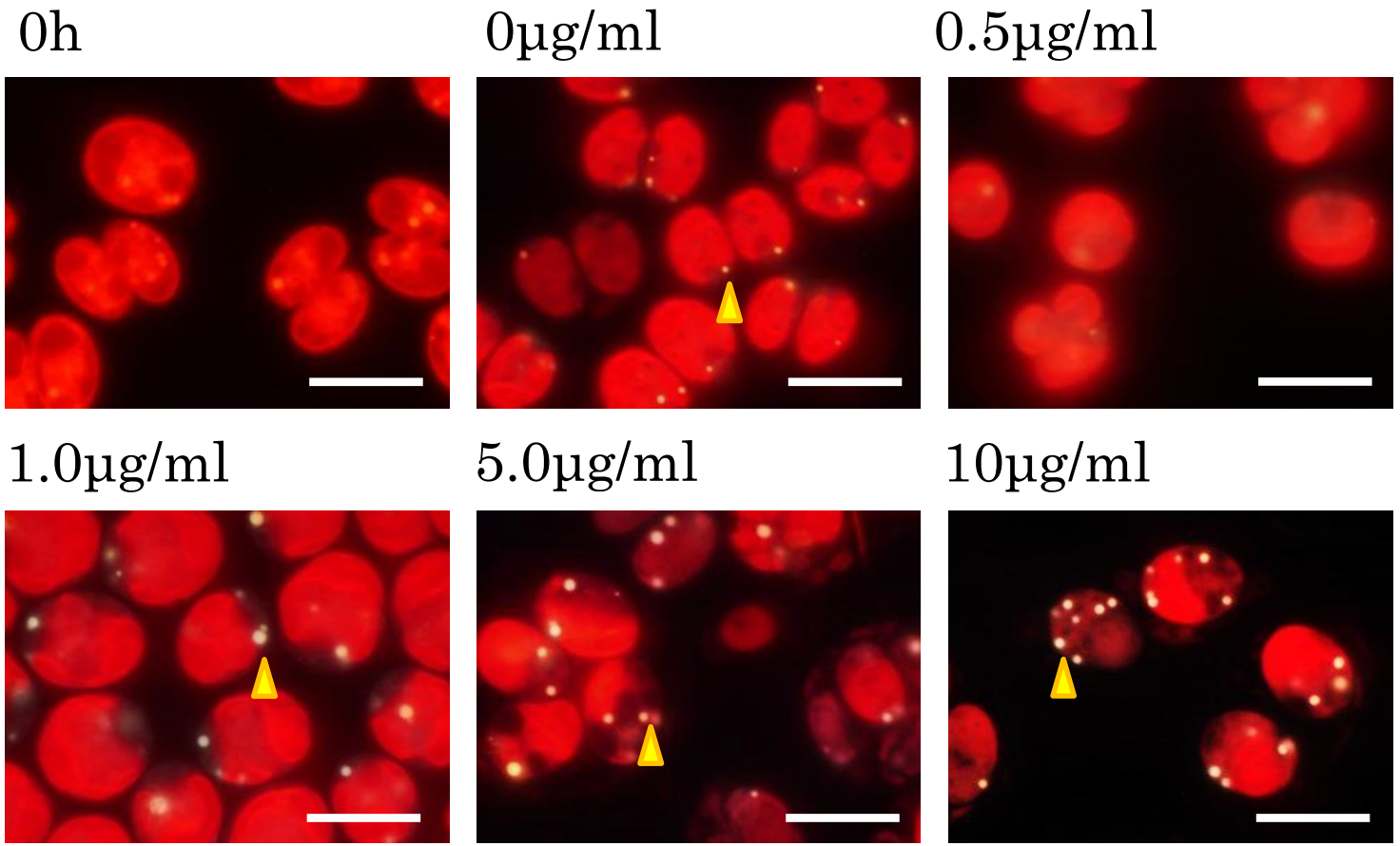


Fig. 3-11. Effect of cyclohexymide on LDs production in TAP 24h. Cyclohexymide was added after culture refreshing by each concentration, inhibits nuclear protein syntheses by interrupting transcription.

Table 3-1. Number of lipid droplets (LDs)

	0μg/ml	10μg/ml
Number of ODs	0.93±0.65	3.69±1.51*

* P<0.05 n=30

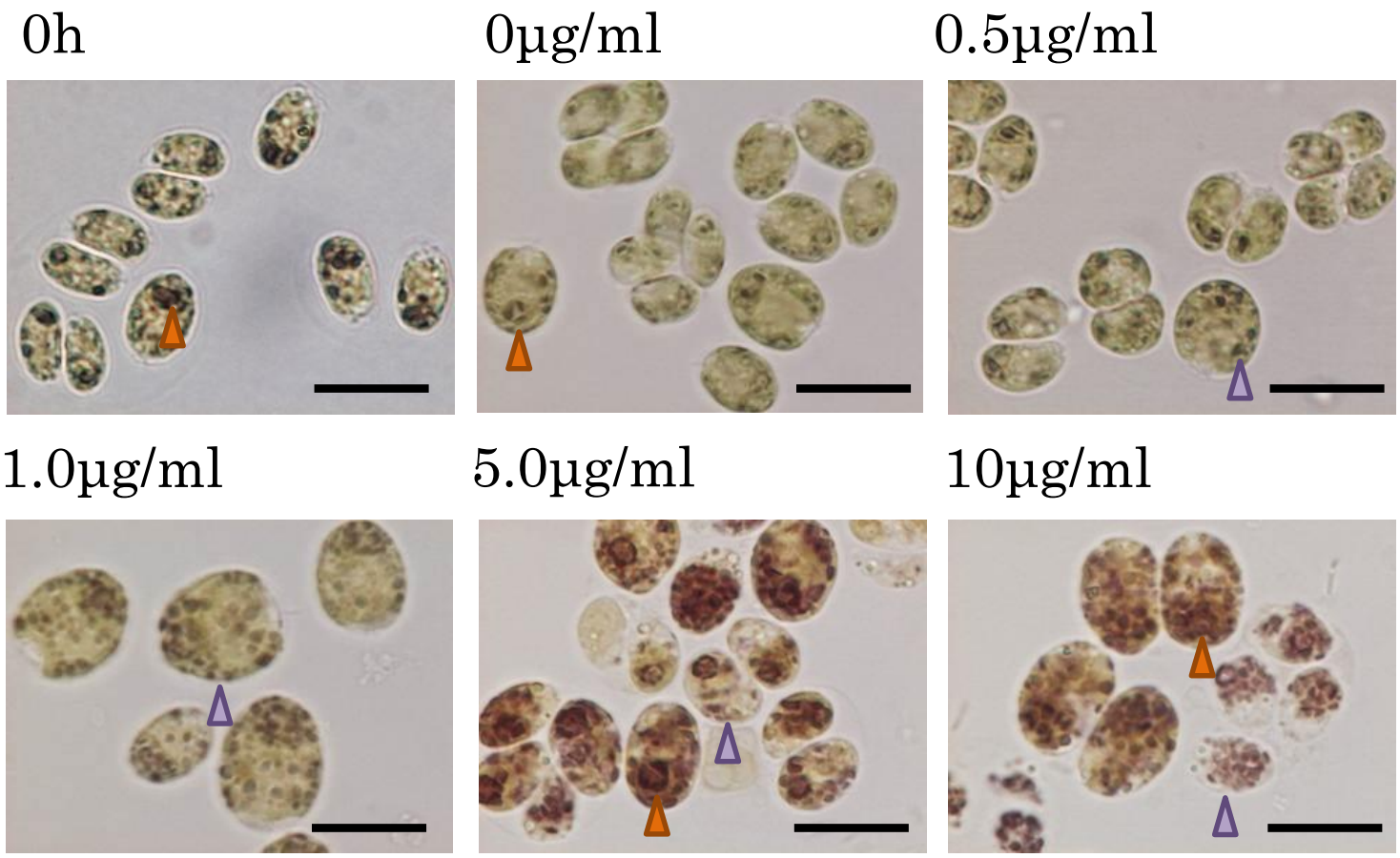


Fig. 3-12. Effect of cyclohexymide on starch production in TAP 24h. Pyrenoid stach : brown triangles, stroma starch : purple tiangles. Starch was stained by 0.1% I_2/1% KI solution.

3-4 Discussion

Adding CHI, cerulenin, and DCMU suppress TAG accumulation under S-starved condition. Thus nuclear coded protein translation, de novo FA synthesis, and photosynthesis were to be necessary for TAG accumulation. These results were accorded with the report of in -N condition (Fan et al., 2011). DGATs are nuclear coded proteins, so that these gene translations led DGATs protein activity and contributed TAG synthesis promotion. Furthermore, celrurenin prevents TAG enhancement, this result suggested that de novo FA synthesis was important for TAG accumulation. However, Ramanan et al reported that genes in FA synthetic pathway were down-regulated under -N condition (Ramanan et al., 2013). The author also examined FA synthetic gene expressions in -S, -N, and -P conditions, but didn't reach a positive result that shows FA gene up-regulation in each stress condition. Therefore, it is not necessary to promote FA synthesis for TAG accumulation. FA synthesis may undergo voluntary by surplus carbon and chemical energy source in the cell. These energy sources (also carbon source) were caused by photosynthesis, so that adding DCMU prevented TAG accumulation. In addition, the result of dark-S denied participation of light signal mechanisms. Cells use TAG to grow without photosynthesis under dark condition (see dark+S). -N-P double stressed condition increased LDs in Fig. 1-3, but cells under -S-N stressed condition didn't accumulate TAG. It would be too stressed to grow and accumulate TAG for *C. reinhardtii*.

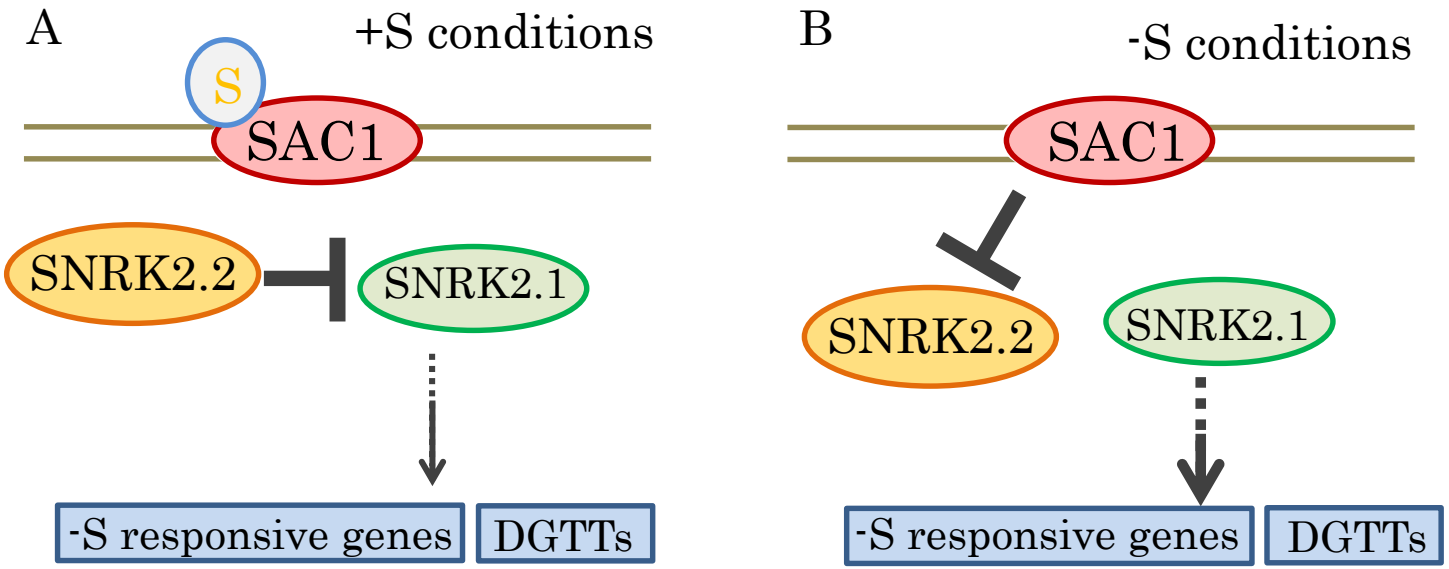
Cells were more hypertrophic under -S and -N condition than usual growth (Fig. 1-3). It was considered that cells withhold cell divisions. Therefore the author examined the relationship between cell division and TAG accumulation. Amiprophos-methyl inhibits (APM) tubulin synthesis and prevents cell division. As the result, there was no remarkable increasing in APM+S in *C. reinhardtii*. However, cooperating with -S condition, the cells showed high TAG increasing as 7.2-fold relative to initial level. The case of without APM was 3.3-fold increase. It is necessary to confirm these results, but other studies support the possibility that abnormal cell division would contribute TAG synthesis. Green alga *Chlorella* increased TAG content by high pH stress. It was insisted that by abnormal cell cycle leads change of lipid metabolisms (Gukert and Cooksey et al., 1990). Temperature dependent cell division mutant in *C. reinhardtii* increased LDs (Yao et al., 2012). Relationships between cell division and TAG accumulation may affect carbon flow (describe below).

Model of sulfur responsive gene expressions by SAC system was shown in supplemental Fig. 3-a. Sulfur deficiency responsive genes such as *aryl-sulfatase (ARS)* are suppressed by SNRK2.2 during SAC1 which function as sensor of sulfate combined with sulfur (A). Since sulfur detached from SAC1 under S-deficient conditions SAC1 inhibits SNRK2.2 and caused -S responsive gene expressions by SNRK2.1. Thus this mechanism explains phenotypes of *Δsac1* as a less sulfur responsible mutant and of *Δsnrk2.2* which expresses sulfur responsive genes

fully in general conditions. Cell growth under -S condition in these SAC mutants corresponded to the characteristics (Fig. 3-2). SNRK2.1 mutant shows less ARS activity and high sensitivity to S-deficiency. These characteristics are also seen in *Δsac1*. Meanwhile, *Δsnrk2.2* showed higher growth than WT and complemented strain under -S conditions. It would be advantages of high S-deficiency responsive gene expression in the cell.

LDs and TAG were included these SAC mutants. Quantitative FA analysis by GC gave interesting results that high TAG content in *Δsnrk2.2* and *Δsac1/SAC1* (Fig. 3-5). High TAG content in *Δsnrk2.2* was contributed by gene expressions and cell growth. Firstly, *Δsnrk2.2* showed higher gene expressions of *DGTT*s than WT and *Δsac1* (Fig. 3-6). Secondly, TAG content of the cells shown per the culture depends on cell volume. *Δsnrk2.2* grew more 1.4 and 1.6 -fold than WT and complemented strains under -S condition (Fig. 3-2). By full expressions of sulfur-deficiency responsive genes gave high acclimation for -S condition to cells, and caused high TAG volume in the culture. Since *Δsac1/SAC1* mutant was inducted *SAC1*gene to *Δsac1*, this mutant may contain more *SAC1* gene copies. Therefore, *Δsac1/SAC1* mutant suppress SNRK2.2 hard. Furthermore, Fig.3-6 indicated important information that *DGAT*s may be under control of SAC mechanisms. Especially *DGTT*s were up-regulated under -S condition, and contributed TAG accumulation. *DGTTs* were controlled by SAC1 positively and SNRK2.2 negatively. A study on nitrogen responsive regulator (NRR) as the other stress responsive control factor was reported. *Δnrr* mutant reduced TAG content to 52% (Boyle et al., 2012). The results of SAC mutants may help the understandig of the mechanism of DGAT gene regulation.

TAG accumulation in ARG assimilation mutant, *Δarg9*, indicated that suppressing of protein synthesis enhanced TAG content (Fig. 3-7, 8). Less TAG content than other -S, and -N conditions was by high sensitivity to ARG deficiency. TAG content per TLs was reduced by ARG addition but that relative to liquid culture was maintained until 120h. This result supported that cell growth affect TAG volume in stress conditions such as -ARG and RAD condition.



Supplemental Fig. 3-a. Model for S deprivation–responsive gene regulation. –S responsive genes are repressed by SAC3 under S replete conditions (A). SAC1 blocks SNRK2.2 (SAC3) inhibition of SNRK2.1, and causes full expression of S-responsive genes. Figure was quoted from GonzaLez-Ballester et al., 2012 and add DGTTs.

General Conclusion

Studies on algal TAG have been developed rapidly in recent days, and especially analyses under N-starved conditions are major method for understanding TAG accumulation in green algae. However, there remains to be clarified about mechanisms of TAG accumulation under stress conditions. In this thesis, the author investigated TAG accumulation under S-starved conditions and compared with those during N- or P-starvation. As a result, it was considered that TAG accumulation depended on the gene expressions and the flow of carbon metabolism. TAG content showed differently under various conditions, and it was highly accumulated both -S and -N conditions. Up-regulations of *DGTT* genes and repression of protein syntheses were involved coincidentally. Moreover, S-starved cells were also increased in the mRNA levels of genes in PA synthetic pathway so that lipid synthesis under S-starved cell was distinct from that of under N-starved ones. Semi-quantitative gene expression analyses did not provide positive evidences of sustainment of higher TAG accumulation under -N condition than that of under -S conditions. The reason was considered that under -N conditions, lipid syntheses may be converged on TAG synthesis and protein synthesis was limited hard.

DGATs are considered to limit TAG synthesis in Kennedy pathway. *C. reinhardtii* possessed five DGTT proteins which were classified into three groups by molecular-phylogeneticaly. Farther studies are more needed to reveal functional differences in these gene-proteins, but an important result was shown with SAC mutants. DGATs seemed to be controlled through SAC regulating system. In particularly, DGTTs were likely to be induced by sulfur-deprived response. This result suggested that TAG accumulation was a part of -S response through the system, and that TAG play a role of cell protection from unfavorable stimulation under stress conditions.

Furthermore, the author showed a behavior of RAD in *C. reinhardtii* and TAG accumulation in *C. kessreli* under -S conditions. *C. reinhardtii* had high sensitivity to RAD conditions, but TAG accumulation was found in the cells. Both dehydration and nutrient-deficiency stresses would induce TAG accumulation. As to *C. kessreli* showed higher growth rate and TAG production under stress conditions than *C. reinhardtii*. Green algae including *C. kessreli* and *C. reinhardtii* are easy to cultivate so that they would have an ability to produce biomaterial productions. Furthermore, using RAD conditions with solid surface would be expected for more applied systems.

This study would contribute to the basal understanding of TAG accumulation in micro algae for the development of bio-oil production technology.

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Thanks for good circumstances.
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Have a dream
And
Enjoy my Life

December 1 2013
Atsushi Sato